Sexual selection in marine plankton

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PhD Thesis by

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“I don't know the question, but sex is definitely the answer.”

Woody Allen

Preface

This thesis was submitted as part of the requirements to fulfill the Doctor of Philosophy Degree (PhD) at the Technical University of Denmark (DTU). The research was carried out at the National Institute of Aquatic Resources, Technical University of Denmark (DTU Aqua) in Charlottenlund under supervision of Professor Thomas Kiørboe. The PhD study also included a research stay at the Section for Marine Living Resources, DTU Aqua, Silkeborg and the research unit Marine Ecology, IFM-GEOMAR, Kiel. This research was funded by DTU, DTU Aqua, and the PhD program SLIP. Additional research funds were provided by Direktør, Dr. Techn. A.N. Neergaard og Hustrus foundation, for which I am very grateful, without this founding the genetic analysis couldn't have been performed. I am further grateful for two travel grants from Otto Mønsted foundation for going to Silkeborg, Kiel and New Orleans.

The overall objective of this PhD project was to examine the presence of sexual selection in marine copepods. I combined different approaches including a series of laboratory studies and genetic parentage analyses to broaden our view on reproduction, mate choice and mate competition in the marine important organism - *Temora longicornis* (Copepoda, Calanoida). Halfway into the project we decided to integrate traditional incubations experiments with genetic work. We joined forces with Einar Eg Nielsen and his group in Silkeborg and Thorsten Reusch, Kiel and here the story about development of microsatellites and paternity testing of *T. longicornis* offspring began. The road has not been easy and a lot of work has gone into identify and characterize the microsatellites. They have not always behaved as we wanted them to, however all the effort has resulted in novel insight in the mating behaviour of this important marine species.

During this journey I have been truly blessed with the help and patience of many people around me. First and foremost, I would like to thank my supervisor **Thomas Kiørboe**, for being who he is. You are my inspiration and motivation on how I should be as a scientist. You have pushed and motivated me all through the process. Even though we have been physically apart several times during the last couple of years, you have always been there for me when I needed it. But you have also been able to let me go and find out things by myself when needed. You have a remarkably ability to know when to push and when to let go, which has strengthen me as a scientist. It has been a truly inspiring journey over the last five years and I hope that I am now ready to stand on my own feet. Also, a great thanks to **Einar Eg Nielsen**, for introducing me to the genetics world and for being willing to move outside the fish world. I have never doubted your support and interest in the project and I have always been met with an open door and a smile. I thank you both for guiding me on my way in science. Likewise, I am greatly grateful to **Dorte Meldrup**, without you, all the genetic work could

not have been done; you have helped me all the way though the laboratories work. I really appreciate the amount of hours you have spent in the trying to get the microsatellites to work. You and Einar have always made me feel welcome in Silkeborg. I would like to thank **Thorsten B. Reusch** for giving me the opportunity to come and visit his group in Kiel and for being helpful in designing the microsatellites. Great thanks to **Uffe Høgsbro Thygesen** for excellent help with statistical models and analysis.

I would like to thank all my collaborators and colleagues who have contributed important ideas and support for my research. In particular, I want to acknowledge the much appreciated help from **Jan Heuschele** and **Sara Ceballos**. You have generously shared your expertise in the lab and *in silico*, I am grateful for sharing good times and exciting discussions; you have been my steadfast support during this PhD study. Especially, I will always remember our two weeks exploring the North Sea. **Jan**, I appreciate all the hours you have spent listing to my sometimes messy thoughts on both science and life while trying to keep track on what I actually is trying to say. I am grateful for all your help with putting this thesis together and I am going to miss our daily discussions about life and science. **Rodrigo Gonçalves** for being my "*alma gemela*" and for becoming my friend; I have really enjoyed the time with you by my side and I hope that our lives will cross paths again. My office mates **Karen**, **Signe**, **Mette**, **Sanne**, **Christina**, **Sofia**, **Julie**, **Evandro**, **Arief**, **Daniel** and **Torkel**: thanks for good coffee (30 seconds plus four minutes, right Mette!), social intercourse and discussions. You have made my day at the office so much more enjoyable and I really valued the work-related discussions as well as the personal ones. Further, I want to thank **Torkel Gissel Nielsen** for taken me under your wings and included me as a part of your group.

This PhD is a culmination of a long education through which I have been privileged to work with a long list of exceptionally talented and inspiring teachers. I would in particularly like to thank my MSc advisor **Benni Winding Hansen** for introducing me to the world of copepods and for believing in me.

Rikke Riis Livoni, I owe you great thanks for moving outside your own field to give me your good and firm comments on my PhD thesis. **Kristine Klinkhammer Adler-Nissen**, I am very grateful for your support all the way - you have helped to increase the quality of my free time. A special thanks to my **family**, **brothers** and special my **mom** and **dad**. **Mom**, for always being by my side, you have always believed in me and your encouragement has been unlimited. **Dad**, to whom I would like to dedicate this work, you will always be in my heart.

Last, but not least, thanks to my husband **Carsten Winding** for always being there. Thanks for putting up with me through this - I could not have done it without you. Your support is priceless and I cannot thank you enough. I am grateful to have you in my life. My most indisputable greatest motivating source in life: **Trine**, **Freya** and **William**; you make me smile, you remind me what life is about – you have my endless love.

Thank you all!

Copenhagen, Marts 2014

Mie Hylstofte Sichlau

Thesis summary

Copepods are among the most abundant metazoans on the planet and play an important role in the marine food web. Many aspects of their ecology have consequently been studied, including details of their reproductive biology and mating behaviour. Sexual selection, the part of evolution which selects for increased mating and fertilization success, is an important evolutionary process, with consequences at individual, population and species level. Yet very little is known about the significance of sexual selection for the evolution and ecology of this key group of animals.

The presented thesis used behavioural studies and genetic parentage analyses to examine the fertilization status and occurrence of mate choice and polygamy in the copepod *Temora longicornis* (Copepoda, Calanoida). The overall objective of my PhD project was to examine the intensity and direction of sexual selection in *T. longicornis* and elucidate the role of sexual selection for the behaviour of individuals, the dynamics of populations, and the functioning of the pelagic ecosystem. I wanted to address the following overarching questions: *i*) What is the fraction of fertilized females in field and laboratory populations? Is it influenced by adult abundance, male mating capacity and the environment? *ii*) Is mating in *T. longicornis* random, or do some individuals have a higher-than-average chance of fertilizing or being fertilized? *iii*) Which traits control mating in *T. longicornis*? Are body size and age significant factors influencing male fecundity (mating rate and sperm production) and female reproductive fitness? *iv*) Does multiple mating lead to multiple paternity? What is the effect of multiple mating on the female's reproductive output as well as the longevity of the individual?

The thesis is divided into 5 chapters that report on different efforts to address these objectivities. It opens with a general introduction and synopsis that lays out the context for the research, summarizes the main findings and discusses perspective for future research (Chapter 1). In Chapter 2 we studied female fertilization status in North Sea summer populations and in laboratory cultures of *T. longicornis*. The study showed that the fractions of fertilized females in both field and laboratory populations were much smaller (< 50%) than predicted by a theoretical model that assumes random mating. Such low fertilization rates are normally related to environmental factors such as poor food or low densities, which we could not confirm in our experiment. Male density was negatively related to fertilization rate, and a large fraction of males did not mate in laboratory incubations. This led to Chapter 3, where we investigated age- and size-dependent reproductive performance (egg and sperm production, mating success) in *T. longicornis*. We found that ageing effects were evident: mortality rate increased with age, and fertility decreased rapidly with age. We also found that several aspects of reproductive performance increased with size in both males and females:

large females produced more offspring than small ones, and large males mated more often, produced larger spermatophores containing more sperm cells and sired more offspring. The study also showed that repeated mating was not only potentially advantageous (e.g. in terms of higher genetic variability) for females, but can come at the disadvantage of increased mortality. Chapter 4 describes the identification and characterization of the six microsatellites primers used in Chapter 5 to do paternity testing of the offspring. To investigate if mating was random or under the influence of sexual selection, we tested which males sired the female's offspring. By establishing paternity, we can precisely quantify the reproductive success of individual males and thus assess how successful particular males have been. Our study clearly demonstrated the occurrence of multiple paternity in *T. longicornis*. This is the first time that genetic polyandry has been documented in a pelagic copepod. Multiple paternity opens up for the possibility of post-copulatory sexual selection, such as sperm competition and cryptic female choice. We further found that mating was non-random, as we identified superior individuals with a higher than average mating success both among females and among males. Some of the variation between individuals could be explained by variation in size and age. Large males were superior to small males in terms of reproductive success, with the larger males (0.8 mm) mating about 3 times as frequent as the smaller males (0.6 mm). In accordance with the findings in Chapter 2, we found that a fraction of the males (8-14 %) in our study never mated during incubations, despite a plentiful supply of females. Only about half of the females in our experiments were fertilized, even though male availability was high and mate encounters not limiting.

The strong size- and age-dependent fertility in this species is conducive to the existence of sexual selection via mate choice for young and large partners, as has been shown in another copepod species. We further suggest that sexual selection, through mate choice or male–male competition could account for low fertilization rates of females in populations of pelagic copepods during some periods of the year.

This thesis suggests that the processes and mechanisms of sexual selection have to be considered when studying reproductive rates in copepod populations.

Dansk resumé

Vandlopper er blandt de mest udbredte organismer på jorden, og de spiller en vigtig rolle i den marine fødekæde. Mange aspekter af deres økologi er derfor blevet undersøgt, herunder detaljer omkring deres reproduktive biologi og parringsadfærd. Seksuel selektion, den del af evolutionen der selekterer for en øget parring- og befrugtningssucces, er en vigtig evolutionær proces, med konsekvenser på både individ, populations og artsniveau. Alligevel ved man ikke så meget om betydningen af seksuel selektion for evolutionen og økologien af denne vigtige dyregruppe.

Denne afhandling anvender adfærdsstudier og genetiske faderskabsanalyser til at undersøge befrugtnings status og tilstedeværelsen af parringsvalg og polygami i vandloppen *Temora longicornis* (Copepoda, Calanoida). Det overordnede mål med mit PhD projekt var at undersøge intensiteten og retningen af seksuel selektion hos *T. longicornis*, og belyse den rolle seksuel selektion har på adfærden af individet, samt på populationsdynamikken og funktionen i det pelagiske økosystem. Jeg ønsker at behandle følgende overordnede spørgsmål: *i*) Hvad er fraktionen af befrugtede hunner i felt- og laboratoriepopulationer? Er fraktionen styret af abundansen af kønsmode individer, hannernes kapacitet til parring og miljøet? *ii*) Er parring i *T. longicornis* tilfældig, eller har nogle individer en større chance for at blive befrugtet eller for at befrugte? *iii*) Hvilke fænologiske træk styrer parring hos *T. longicornis*? Er kropsstørrelse og alder signifikante faktorer der påvirker hannens forplantningssucces (parringsrate og spermproduktion) samt hunnens reproduktive succes? *iv*) Fører gentagne parringer til flere forskellige fædre til afkommet? Hvad er effekten af gentagne parringer på hunnens reproduktive udkast, såvel som på levetiden for individet?

Afhandlingen er delt ind i fem kapitler, som er resultaterne af vores indsats for at undersøge og besvare disse forskellige spørgsmål. Kapitel 1 indledes med en generel diskussion og synopsis, der opridser konteksten for forskningsprojektet, opsummerer hovedkonklusionerne og diskuterer fremtidig forskning. I kapitel 2 undersøger vi fraktionen af befrugtede hunner i sommerpopulationer i Nordsøen, og i laboratoriekulturer af *T. longicornis*. Studiet viser at fraktionen af befrugtede hunner er meget lavere (< 50 %) end forudset af teoretiske modeller der antager tilfældig parring. Sådan en lav befrugtningsrate er normalt relateret til miljømæssige faktorer, såsom lav kvalitet eller kvantitet af føde. Dette kunne vi dog ikke bekræfte i vores forsøg. Densiteten af hanner var negativt korreleret med fertiliseringsraten, og en stor fraktion af hannerne parrede sig ikke i inkubationerne. Dette førte til kapital 3, hvor vi undersøgte alders- og størrelsesafhængig reproduktionssucces (æg og sperm produktion, parringssucces i *T. longicornis*). Vi fandt at individernes alder havde en effekt: mortalitetsraten forøgedes med alderen, og fertiliteten faldt ligeledes hurtigt med alderen. Vi fandt også at flere aspekter af den reproduktive ydeevne blev forøget med størrelsen hos både hanner og hunner: store hunner producerede mere afkom end små hunner, og store hanner parrede sig oftere,

producerede større spermatophorer der indeholdt mere sperm, og de producerede mere afkom. Studiet viste også, at gentagne parringer ikke kun var en fordel (for eksempel i form af højere genetisk variabilitet) for hunnen, men kan være en ulempe på grund af forhøjet mortalitet. Kapitel 4 beskriver identifikationen og karakteriseringen af seks mikrosatellit primers, der bliver brugt i kapitel 5 til at udføre faderskabsanalyse på afkommet. For at undersøge om parring var tilfældig eller under påvirkning af seksuel selektion, testede vi hvilke hanner der var fædre til hunnernes afkom. Ved at etablere faderskab var vi i stand til præcis at bestemme den reproduktive succes af individueller hanner, og dermed vurdere hvor succesful en bestemt han havde været. Vores studie viste tydeligt forekomsten af afkom med forskelligt faderskab i *T. longicornis*. Det er første gang at genetisk polyandri er blevet dokumenteret i en pelagisk vandloppe. Flere forskellige faderskaber giver mulighed for post-kopulativ seksuel selektion, såsom sperm-konkurrence og hunnens eventuelle tilvalg og/eller fravalg af sperm fra bestemte hanner, også kaldet ”cryptic female choice”. Vi fandt endvidere, at parring ikke var tilfældig, idet vi identificerede overlegne individer, både blandt hunner og hanner, som havde højere parringssucces end gennemsnittet. Noget af variationen imellem individer kunne forklares ved variationen i størrelse og alder. Store hanner var overlegne i forhold til små hanner i form af reproduktiv succes, og store haner parrede sig ca. tre gange oftere end små hanner. I overensstemmelse med resultaterne i kapitel 2 finder vi, at fraktionen af hannerne (8-14 %) i vores studie aldrig parrede sig under inkubationerne, på trods af en rigelig tilgang til hunner. Kun halvdelen af hunnerne i vores eksperimenter var befrugtede, selvom tilgængeligheden af hanner var stor og at møder mellem individer af de to køn ikke var begrænsende.

Denne stærke størrelses- og aldersafhængige fertilitet i denne art bidrager til eksistensen af seksuel selektion via positiv selektion for unge og store partnere, som tidligere er blevet påvist i en anden vandloppeart. Vi foreslår endvidere at seksuel selektion via partnervalg eller konkurrence mellem hanner**,** kan redegøre for den lave fertilisationsrate af hunner i populationer af pelagiske vandlopper i nogle perioder af året.

Denne afhandling foreslår at processerne og mekanismerne bag seksuel selektion tages i betragtning, når man studerer reproduktive rater i vandloppepopulationer.

Contents

LIST OF ORIGINAL PAPERS

I. Ceballos, S., **Sichlau, M.H.**, Heuschele, J., Kiørboe, T. (2014) *Low fertilization rates in a pelagic copepod caused by sexual selection?* Journal of Plankton Research 36: 736-742. doi: http://dx.doi.org/10.1093/plankt/fbu021

II. **Sichlau, M.H.**, Kiørboe, T. (2011) *Age- and size-dependent mating performance and fertility in a pelagic copepod, Temora longicornis.* Marine Ecology Progress Series 442: 123-132. doi: http://dx.doi.org/10.3354/meps09402

III. **Sichlau, M.H.**, Reusch, B.H., Meldrup, D., Nielsen, E.E. Kiørboe, T. *Identification and characterization of six microsatellite primers for the calanoid copepod Temora longicornis.* Submitted to Conservation Genetic Resources

IV. **Sichlau, M.H.**, Nielsen, E.E. Kiørboe, T. *Mating success and sexual selection in a pelagic copepod, Temora longicornis*: *Evidence from paternity analyses.* Submitted to Limnology and Oceanography

Co-authored papers not included in the thesis

Drillet, G., Frouel, S., **Sichlau, M.H.**, Jepsen, P.M., Højgaard, J.K., Joarder, A.K., Hansen, B.W. (2011) Status and recommendation on marine copepod cultivation for use as live feed. Aquaculture 315: 155-166.

CHAPTER 1

General introduction and summery of findings

Mie Hylstofte Sichlau

Drawing by Jan Heuschele

1. Introduction

1.1 Background

The oceans cover 70 % of the planet´s surface, and the marine ecosystems including its plankton have great ecological and economic significance. However, our knowledge about the evolution and population ecology of marine plankton (compared to terrestrial systems) is still incomplete. The ocean has been under scientific investigation for centuries and yet our understanding of the driving forces and the underlying structure of pelagic ecosystems, especially of the functioning of planktonic organisms, is still limited.

Sexual selection has been a hot topic in evolutionary ecology over the past 40 years, with studies mainly on terrestrial organisms and larger aquatic animals. Researchers have only recently realized that sexual selection may be also important for the evolution and population ecology of small plankton organisms (Titelman et al. 2007). Copepods, one of the most abundant metazoans on the planet (Humes 1994, Turner 2004) are an important part of plankton and play a crucial role in the marine food web. Many aspects of their ecology have consequently been well studied, including details of their reproductive biology and mating behaviour (Uchima & Murano 1988, Doall et al. 1998, Yen et al. 1998, Weissburg et al. 1998, Buskey 1998, Bagøien & Kiørboe 2005a, Titelman et al. 2007, Dur et al. 2011, Seuront 2013, Heuschele et al. 2013). Yet, very little is known about the significance of sexual selection for the evolution and ecology of this important group of animals (Titelman et al. 2007). The past neglect has been mainly due to two factors: Sexual signals among small organisms are difficult to visualize (unlike e.g. the feather plumage and courtship behaviours of birds), and secondly, the implicit assumption that mating is encounter-limited in copepods (Titelman et al. 2007), thus, not subject of sexual selection. However, recent studies have shown that mate encounter rates in pelagic copepods are typically high (Kiørboe & Bagøien 2005) and that sexual selection may operate via mate choosiness or mate coercion, with both males and females preferring to mate with large and young partners (Ali et al. 2009, Ceballos & Kiørboe 2010, Chapters 3 and 5).

1.2 Focus of the thesis

The present thesis performs behavioural studies and molecular analysis to examine the fertilization status and the occurrence of mate choice and polygamy in the copepod *Temora longicornis* (Copepoda, Calanoida). The overarching aim has been to examine the intensity and direction of sexual selection and elucidate its role on the biology of individuals, the dynamics of populations, and the functioning of the pelagic ecosystem.

In the synopsis of my PhD thesis, I will discuss the background and motivation for carrying out this work, briefly describe the methodology used, provide a summary of the findings and discuss the major conclusions and future perspective of this study.

2. Sexual selection

Darwin (1871) was the first to understand the existence and importance of sexual selection; he realized that not all differences between males and females were due to their specialized roles in sexual reproduction. Sexual selection arises from variance in mating success, caused by some individuals having an advantage over others in acquiring mates and in general acts through mate choice, intra-sexual competition for mates, and mate coercion. Sexual selection drives the evolution of traits that increases fertilization success (Andersson 1994, Kokko & Jennions 2008) and makes many organisms go to extremes to impress and attract the other sex: peacocks maintain elaborate tails, elephant seals fight over territories and some species deliver persuasive gifts to enhance their reproductive success (Darwin 1859, Darwin 1871, Le Boeuf 1974, Andersson 1994, Vahed 1998) and often sexual selection comes at the cost of decreased longevity. For example, conspicuous and colourful feathers are likely to attract predators as well as potential mating partners of the opposite sex (Andersson 1994). The goal of evolving sexual selection strategies arises in gaining the highest lifetime reproductive success relative to others, i.e. a high contribution of fit offspring to the next generation. Sexual selection is an important process for micro- and macro evolution (Schluter 2001) and has proven to be a very strong determinant of the evolution of the morphology, behaviour of individuals, speciation and biodiversity in many species (Emlen & Orin 1977, Andersson 1994, Schluter 2001, Kokko & Rankin 2006).

In the following, the influence of mate choice, polygamy and intra- and intersexual sexual selection and its consequences for the strength of sexual selection are discussed. Figure 1 illustrates the central role of sexual selection and its interaction with many aspects of the ecology and behaviour of *Temora longicornis* that are discussed and studied in this thesis.

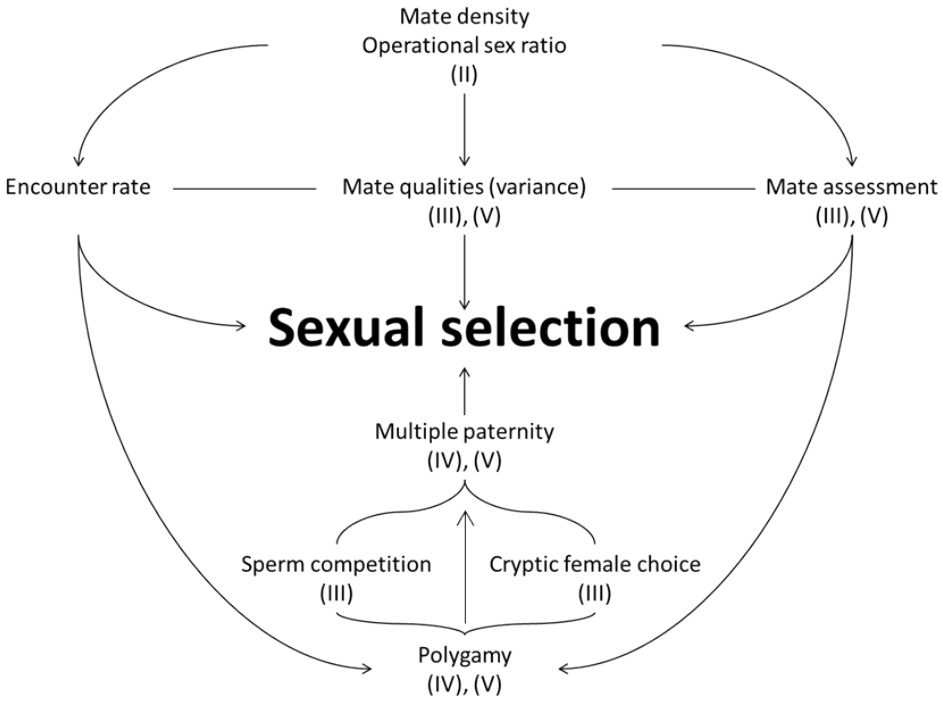

Figure 1: General overview showing the vital role of sexual selection and its interaction with many different aspects of the ecology and behaviour of *Temora longicornis*. Roman numerals refer to the chapters regarding the subject.

Darwin recognized two mechanisms of sexual selection *i*) intrasexual selection, or interaction between members of one sex ("*the power to conquer other males in a battle*," as Darwin put it), and *ii*) intersexual selection, essentially mate choice ("*the power to charm*"), see table 1. Sexual selection can take place both before and after copulation. Pre-copulatory selection includes male competition and female mate choice (Birkhead & Møller 1998). Post-copulatory selection takes place inside the female's reproductive tract and can either be caused by sperm competition between different mating partners or by cryptic female choice and selective sperm utilization (Birkhead & Møller 1998, Birkhead & Pizzari 2002). Due to the simultaneous presence of sperm competition and cryptic female choice hidden inside the female reproductive organs, these post-copulatory processes are difficult to observe and difficult to clearly tell apart. However, they play an important role in sexual selection and therefore research about post-copulatory sexual selection has become an important topic in the last years, especially with the increasing availability of molecular techniques.

	Mating success # matings per lifetime	Fertilization success # offspring sired per mating	Reproductive fitness
Intersexual selection	Female choice	Sperm choice	
Intrasexual selection	Male-male competition	Sperm competition	

Table 1: The different mechanisms of inter- and intersexual selection and their effect on the reproductive fitness.

2.1 Mate choice

Three conditions have to be fulfilled for sexual selection to be an evolutionary success (Kokko & Monaghan 2001, Shuster 2007), and these conditions may change the direction and intensity of sexual selection:

The mate encounter rate must be high enough so that mates can afford being choosy. Mate finding is a challenge for small animals living in a 3-dimensional world (Buskey 1998). Female copepods use chemical and hydromechanical cues to signal their presence and position to males (Tsuda & Miller 1998, Doall et al. 1998, Bagøien & Kiørboe 2005a). Models describing female signal extension and male search behaviours have been developed (Bagøien & Kiørboe 2005a, Bagøien & Kiørboe 2005b) and we now have estimates of the volumes of water males can search for females per unit time (Kiørboe & Bagøien 2005, Choi & Kimmerer 2009). The estimated mate encounter rates frequently exceed the copulatory capacity of the males and the breeding capacity of the females (Kiørboe 2006, Kiørboe 2007, Kiørboe 2008).

There must be a significant cost of breeding. This cost can be characterised as a reduction in the ability to invest in future offspring due, e.g., to breeding-related time expenditure and mortality (Chapter 3). While most breeding costs for females are rather obvious, sperm is normally considered a non-limiting resource and, hence, the cost of breeding is often assumed to be low in males. However, there are indications that the competition for females and the energetic cost of producing spermatophores have larger energetic requirements than commonly believed (Dewsbury 1982). The cost of a spermatophore is much greater than the individual spermatozoa. *Temora longicornis* has a relatively low spermatophore production, < 1.5 spermatophores per day (Ceballos et al. 2014), suggesting that spermatophore production may be expensive. Multiple mating decreases the longevity of both sexes in *T. longicornis* (Chapter 2) and in another copepod species, *Oithona davisae* (Ceballos & Kiørboe 2011).

There must be differences among mates that can be perceived by the partner. Video-observations of courtship behaviour and observed female 'resistance' suggest a mechanism of partner assessment (Kiørboe et al. 2005). Copepods probably assess mate attractiveness and quality from hydromechanical and chemical cues and we have demonstrated that both males and females prefer to mate with large and young partners (Ceballos & Kiørboe 2010, Chapters 3 and 5), indicating the presence of pre-copulatory mate choice.

2.2 Mate choice in copepods

It has been shown for a number of organisms that larger individuals generally produce greater numbers of gametes (MacDiarmid & Butler 1999, Lehmann & Lehmann 2009, Gasparini et al. 2010). Egg production rates increase with female size in copepods (Ceballos & Kiørboe 2010, Kiørboe & Hirst 2008, Chapter 3 and 5) and male size correlates with spermatophores size (Ceballos & Kiørboe 2010, Chapter 3). There can be great variance in adult body size within copepod species and body size has shown to be a heritable trait (McLaren 1976, McLaren & Corkett 1978, Dam & Peterson 1991, Arendt et al. 2005). For both sexes there may be an advantage in mating with large partners, given that the offspring will have large offspring themselves. The age of the males is just as important as body size for copepods in deciding to accept or reject a potential partner (Ceballos & Kiørboe 2011, Uchima 1985, Chapter 3 and 5). In *Temora longicornis* we found that mortality increased and fertility decreased rapidly with age (Chapter 3 and 5), indicating an 'age penalty'.

2.3 Operational sex ratio

The direction and intensity of sexual selection are often evaluated on the basis of the operational sex ratio (OSR). The OSR is the average ratio of fertilizable females to sexually active males at any given time (Emlen & Orin 1977, Kvarnemo & Ahnesjo 1996) and is a main determinant of the opportunity for sexual selection (Andersson 1994, Kvarnemo & Ahnesjo 1996). The sex with the highest parental investment becomes limiting and thus evolves choosiness, while the non-limiting sex will compete for mates. In a male-based OSR, females can become more selective, and males tend to be more competitive. In a female-based ORS the male have less competition and can mate with more females, the females also tend to be less discriminative (Dur et al. 2012). The OSR predicts the degree of competition for mates and therefore is thought to correlate with the strength of sexual selection (Kvarnemo & Ahnesjo 1996, but see Klug et al. 2010). This feeds back into the evolution of the mating system, which can affect the primary sex ratio, thus partially determining the OSR (Andersson 1994).

2.4 Cost and benefits of sexual selection

Female choice is an extensively studied phenomenon and is now widely accepted as an important component of sexual selection (Andersson 1994), however the involved mechanisms are still being debated (Grafen 1990, Kirkpatrick & Ryan 1991, Kirkpatrick & Barton 1997, Kokko et al. 2002, Kokko et al. 2003, Cordero & Eberhard 2003, Cordero & Eberhard 2005, Kokko et al. 2006, Kokko et al. 2007). Bateman (1948) was the first to show that male fitness increases linearly with access to mates whereas female fitness in contrast decreases with increased mating opportunities. The reason for this is that female reproduction is generally more limited by factors such as egg production and resources required for parental care (Cluttonbrock & Parker 1992). Both sexes can gain direct and indirect benefits from selective mating, and thereby increase the lifetime reproductive success of the individual (Andersson 1994). However, it can also be costly for all involved partners and mate behaviours such as mate coercion and mate competition can be expensive for both the female and the male. Direct benefits for females in different taxa include increased fertility, paternal care, nuptial gifts and females can also obtain other material benefits from sperm fluid, such as defensive chemical compounds that they either can use themselves or transfer to their offspring as in the case of the moth *Utetheisa ornatrix* (Gonzalez et al. 1999, Iyengar et al. 2001). Indirect benefits also increase the lifetime reproductive success of the female by providing compatible genes to her offspring. Indirect benefits may arise from the specific alleles of a male and the female choose for “good genes”, by mating with males with traits that indicate heritable viability of the father (Houle & Kondrashov 2002). If males display a trait, e.g. the quantity of pheromones, that correlates with their viability and fitness, and the viability is heritable, then the females mating with males with a high quantity of pheromones will achieve genes with high viability to pass on to their offspring. This is the case of the parasitic wasp *Nasonia vitripennis* where the quantity of male sex pheromone is correlated to the quality of the male (Ruther et al. 2009). According to Fisher (1930) females are expected to choose males with traits that are likely to increase the attractiveness of offspring (i.e. sexy sons hypothesis), which in turn is expected to increase female fitness. Fisher (1930) predicted a self-sustained process, whereby genes for a female preference and “good” genes become genetically correlated (reviewed in Kokko et al. 2006). Indirect benefits also include the choice for a partner with a compatible set of alleles, so that the offspring will have an optimal number and assortment of alleles as seen in sticklebacks (Reusch et al. 2001).

3. Polygamy

Polygamy is defined as a mating pattern in which a single individual mates with more than one individual of the opposite sex. Polygamy occurs across a wide range of environments and taxa, including insects (Arnqvist & Nilsson 2000), birds (Birkhead & Møller 1995), mammals (Gomendio et al. 1998), mollusks (Dupont et al. 2006), fish (Feldheim et al. 2002, Dibattista et al. 2008, Barbosa et al. 2010), and reptiles (Laloi et al. 2004, Uller & Olsson 2008). During the last decades the development of new molecular techniques has changed our understanding of mating systems in nature, and species that were thought to be monogamous have revealed moderate to high levels of polygamy (Birkhead & Møller 1998, Simmons 2005, Parker & Birkhead 2013). Males benefit by multiple mating through increased reproductive success (Bateman 1948, Arnqvist & Nilsson 2000). For females, the adaptive significance is less clear, as in most cases it does not result in an increased number of offspring (Andersson 1994, DiBattista et al. 2008).

3.1 Polygyny

Males are expected to be promiscuous; because male reproductive success is typically directly related to the number of inseminated females. However, in polygamous species, the successful male must be able not only to inseminate multiple females, but also to outmatch sperm from previous males, to avoid sperm displacement by future males and have a high quality sperm, so the female will choose his sperm over others to fertilize her eggs (Eberhard 1996, Simmons 2001a). Consequently males in polygamous species are subject to intense pre- and post copulatory sexual selection.

3.2 Polyandry

The adaptive significance of female promiscuity is less clear than in the case of polygyny. Female multiple mating may be male driven, and thus just a result of mate coercion. However, in many species females actively promote mating from multiple mates, for example in the hide beetle, *Dermestes maculatus* the female is more likely to mate with a new male than with one she already has mated with (Archer & Elgar 1999). The puzzle of polyandry is more perplexing when one considers the often considerable costs to females of mating as in the case of *Temora longicornis*, where repeated mating led to higher mortality for the female (see Chapter 3). The costs of polyandry can be manifold and include wasted time and energy, increased risk of predation and disease, potential damage caused by male seminal fluids and copulatory organs, and even death (Keller & Reeve 1995, Eberhard 1996).

One possible explanation for polyandry is that it enables females to mate with several genetically superior individuals, thus enhancing the genes passed on to their offspring. In many species, females mate with more than one male to receive direct material benefits (e.g., fertilization assurance and material benefits provided in spermatophores) and indirect genetic benefits (e.g., higher offspring diversity and higher offspring viability) and to avoid genetic incompatibility and inbreeding (Arnqvist & Nilsson 2000, Birkhead 2000, Jennions & Petrie 2000, Arnqvist & Rowe 2005, Simmons 2005). If males differ in heritable traits, polyandrous females also have the opportunity to choose between the sperm of several males by cryptic female choice (Eberhard 1996).

In pelagic copepods, polyandry is often observed both in laboratory and field populations, as evidenced by females carrying multiple spermatophores, see figure 2 (Katona 1975, Jacoby & Youngbluth 1983). Females of *T. longicornis* are often seen carrying several attached spermatophores simultaneously (up to 70 attached spermatophores in the laboratory, pers. obs.), implying that they frequently re-mate, even after been fertilized, see figure 2. Each spermatophore represents a mating event and hence the presence of multiple spermatophores is direct evidence of multiple mating events. However, many females obtain enough sperm from one mating (Ridley 1988, Chapter 3). It is unknown if the semen of *T. longicornis* contains other substances than sperm cells.

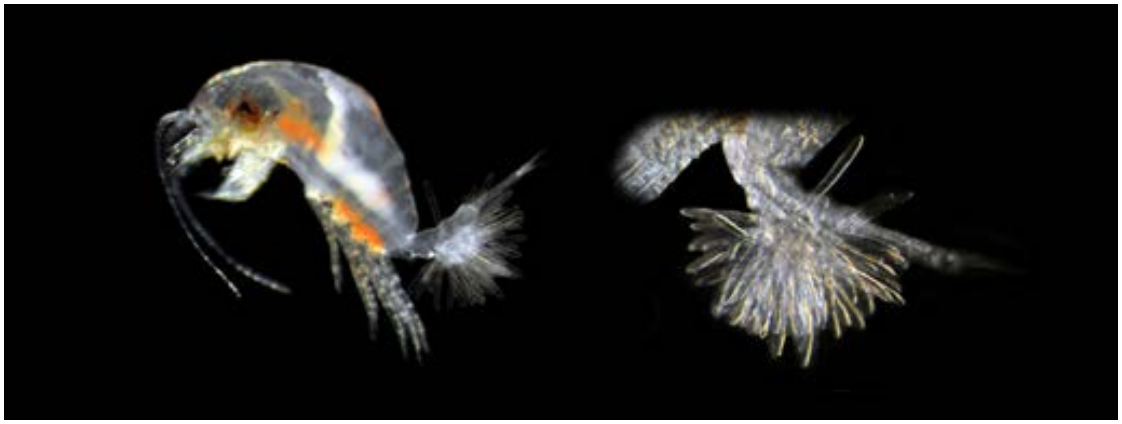

Figure 2: *Temora longicornis* female with multiple attached spermatophores.

3.3 Direct benefits of multiple mating

Females may benefit directly from additional matings by gaining increased access to supply of sperm. Sperm is clearly beneficial to virgins. However already mated females would only benefit from extra matings if they did not received sufficient sperm from the first mating or are unable to store sperm and therefor need a steady supply of sperm during their reproductive life (Ridley 1988, Pitnick & Markow 1994, Choe & Crespi 1997). From an evolutionary stand point, a male should produce sufficient sperm to fertilize all the females' egg to best enhance his fitness. However, sperm allocation by males is a subtle trade-off, which sometimes has to be optimised for female quality and number of mates, whilst balancing the risks of sperm competition (Simmons & Kvarnemo 1997, Galvani & Johnstone 1998, Wedell et al. 2002).

3.4 Indirect benefits of multiple mating

Indirect benefits can be categorized into the following four classes: *i*) "*Trading Up"*, where females mate sequentially with males of improving quality; *ii*) "*Bet Hedging"*, where females produce broods of multiple paternity and benefit from a heterogeneous brood that is better adapted to an unpredictable environment; *iii*) "*Sperm Sorting*", where females benefit from either 'cryptic' choice, selecting from diverse sperm, or by invoking sperm competition, and thus producing sons with superior sperm (if sperm ability is heritable); and *iv*) "*Incompatibility Avoidance*", where females vary at the genetic or cytological level for their preferred mate partner, and polyandry increases the odds of finding a compatible partner.

3.4.1 Trading up

In species that perform multiple mating, it has been suggested that firstly the female should mate unselectively and then secondly "*trade up*" by improving the mate quality in the following matings by accepting additional copulations only from superior mates (Jennions & Petrie 2000). "*Trading up*" is consistent with previous models of sexual selection and explains polyandry as an outcome of females accepting additional mates in a sequential manner when they offer an enhancement on previous mates (Thornhill & Alcock 1983, Kempenaers et al. 1992, Graves et al. 1993, Hasselquist et al. 1996, Petrie & Kempenaers 1998, Jennions & Petrie 2000). When females have access to several males sequentially, they have to decide if they should remain unmated versus mating with males of low quality. This balance depends on the encounter rate with males. When the encounter rate with males are low, a virgin female may do better to accept any mating opportunity regardless the quality of the male, compared to remain unfertilized the rest of her lifetime (Kokko & Ots 2006, Lehmann 2007).

3.4.2 Bet hedging

'Bet hedging' is a generalist strategy, where females produce heterogeneous offspring as an adaptation to uncertainty, e.g. an unpredictable environment (bet-hedging under the genetic diversity hypothesis) or imperfect mate choice when searching for the "good genes" (bet-hedging under the good genes hypothesis) (Watson 1991, Stockley et al. 1993, Yasui 1998, Yasui 2001). In a changing and unpredictable environment, fit genotypes may be different between generations, so the female cannot predict which genes which will be successful in the next generation (Yasui 2001). Bet hedging leads to a decrease in temporal fitness variation at the expense of a reduction in average fitness.

3.4.3 Sperm competition

Sperm competition is the main form of male-male, post-copulatory competition in the period between mating and eggs fertilization (Birkhead & Møller 1998). Sperm competition arises when fertile sperm from two or more males co-occurs within a female reproductive tract and competes to fertilize her eggs (Parker 1970). Sperm competition is a remarkably powerful selective force, which has led to a variety of behavioural, morphological and physiological adaptations in both sexes that either enhance the competitive advantage of a male's sperm or counter the sperm of competitors (Birkhead & Møller 1998, Birkhead et al. 2009). The presence of sperm from multiple donors may allow females to choose their mates cryptically, giving the female an additional opportunity to screen for the best mate (Eberhard 1996). Cryptic choice allows females to accept copulations without necessarily having to use the received sperm. Therefore, if males use coercive mating tactics, females may be able to accept copulations from unwanted males and thus avoid the costs (e.g. energy, physical injury) to escape from the male, whilst avoiding using their sperm.

3.4.4 Incompatibility Avoidance

Genetic incompatibility depends on how the genes of females and males interact and the female will choose males with genes there are compatible with her owns. Therefore optimal male quality depends on the specific female accessing him (Zeh & Zeh 1996, Zeh & Zeh 1997, Tregenza & Wedell 1998, Tregenza & Wedell 2000, Simmons 2001b). Preference for genetic compatibility has been found to be especially important in the evolution of the major histocompatibility complex (MHC). Mating preference which were MHC-dependent have been demonstrated in humans, mice and sticklebacks (reviewed in Penn & Potts 1999).

4. Male coercion and sexual conflict

The goal of both sexes is to optimize their respective reproductive success, however their genetic interest are not identical, resulting in sexual conflict (Parker 1979). The conflict that occurs between an unwilling female and a willing male is perhaps the most important form of sexual conflict for evolution (Lessells 2006). The most successful males are not necessarily the most desirable, and the simplest explanation for polyandry could be that it has no adaptive value for females and is just a result of male coercion and power (Thornhill & Alcock 1983, Halliday & Arnold 1987, Ridley 1990, Rowe et al. 1994). Traits that allow males to increase their reproductive success at the expense of the female's fitness will be positively selected if the female mates with multiple males. If the trait is heritable and the males carrying these traits are more successful in mating, then the trait will be genetically transmitted to the offspring and spread in the population with time (Parker 1979). The evolution of a trait that imposes damage on one sex will lead to the evolution of female resistance (Chapman et al. 2003, Arnqvist & Rowe 2005). Males are certainly able to force copulations in many species (Seymour 1990, Gowaty & Buschhaus 1998, Low 2005), and in copepods male coercion has been found in one species, *Eudiaptomus graciloides* (Ali et al. 2009). Evidence of pre-copulatory conflict can be found in the evolution of 'grasping' traits in males, or other features that allow them to overcome female resistance. Males of copepods have evolved modified antennae that they use to grasp the female during mating.

5. The study organism

Copepods are one of the most abundant organism groups in the world and during their evolution, starting in the Lower Cretaceous, copepods have spread all over the world and have successfully colonized virtually every aquatic habitat of the Planet - from the deep-sea vents, anchialine caves to the Himalayan mountains, becoming well adapted and specialized to very different salinity regimes, from marine and hypersaline waters to continental freshwater bodies, and to a wide range of temperatures from the polar to hot springs waters. In terms of their size, diversity and abundance they are often referred to as "*insects of the sea*".

Figure 3. Illustration of “love life” of copepods: One female (darker individual on top left) has been mated several times and swims through the water with a bouquet of spermatophores attached on the urosome. One couple (mid left) is in the act of mating, while other males are searching for mates and food. Most of the females are unfertilized and are cruising through the water on the hunt for food, while laying out a pheromone trail to attract potential partners.

Temora longicornis is a species common to temperate shelf waters of both northern European and American continents as well as in the North and Baltic Seas (e.g. Barnard et al. 2004, Razouls et al. 2005–2007 available at: http://copepodes.obs-banyuls.fr/en). *T. longicornis* are so called “trail followers”. The female leaves a discrete trail of pheromones, which the male can use to detect and locate the female (Doall et al. 1998, Goetze & Kiørboe 2008). During mate search the male pursues the female from behind with increased swimming speed, and then with coordinated turns and short, quick movements the male tries to capture her by grasping the female with the geniculate right 1st antenna (Watras 1983). After coupling the animals spin rapidly. The female urosome is grasped by the male's right 5th leg and the left 5th leg is used to attach a spermatophore, containing sperm, to the genital segment of the female (Watras 1983). The female and male remain connected for several minutes in this position (pers. obs.), see figure 4. After the male has

successfully attached the spermatophore on the female, the spermatozoa empty into the genital antrum (Mauchline 1998). Spermatozoa of copepods are round and non-motile and are pushed into the female's genital antrum by means of mechanical and hydrostatic by swelling of modified cells located immediately within the wall of the spermatophore (Blades-Eckelbarger 1991).

Figure 4: *Temora longicornis* male and female mating (top) and female with multiple attached spermatophores (bottom).

6. Research objectives

The overall objective of my PhD project was to investigate the presence of sexual selection in the calanoid copepod *Temora longicornis*. We wanted to address the following overarching questions:

- What is the fraction of fertilized females in field and laboratory populations? Is it influenced by adult abundance, male mating capacity and the environment?
- Is mating in *T. longicornis* random, or do some individuals have a higher-than-average chance of fertilizing or being fertilized?
- Which traits control mating in *T. longicornis*? Are body size and age significant factors influencing male fecundity (mating rate and sperm production) and female reproductive fitness?
- Does multiple mating lead to multiple paternity? What is the effect of multiple mating on the female's reproductive output as well as the longevity of the individual?

7. Summary of studies and findings

Below, I briefly outline the main findings from each thesis chapter and discuss how the combined conclusions contribute to our understanding of the ecology and behaviour of *Temora longicornis.* Only the main findings are discussed here, while a more in depth discussion of the results is given within each of the following chapters 2-5.

Chapter 2: Low fertilization rates in a pelagic copepod caused by sexual selection?

This paper is based on data from a cruise to the North Sea in 2010 and subsequent laboratory studies. We assessed the prevalence of fertilization limitation within copepod populations, due to its importance for understanding secondary productivity in coastal food webs. We investigated the fraction of fertilized females in the field and in the laboratory, and found that a large fraction of the females were not fertilized in both populations. We compared this fraction of fertilized females (< 50 %) with predicted values from a theoretical model assuming random mating. We found that the fractions were much smaller than predicted by the model.

Low fertilization rates are normally attributed to detrimental or a poor female diet, the occurrence of sex change and suboptimal temperatures, however we could not relate food availability (chlorophyll *a* concentration in the maximum fluorescence depth), sex ratio nor male mating rate to the low fraction of fertilized females. However, we found that the fraction of fertilized females was negatively correlated with both adult and male density. On average males mated < 1.5 times per day and a large fraction 25-57 % of the males did not mate. The male mating rate was not influenced by the sex ratio, or by female, male or adult abundance in their original population and we did not find any variance between the laboratory and field experiments.

We suggest that mating in *Temora longicornis* is not random and we propose that sexual selection by mate choice, male-male competition and other reproductive behaviours could explain the low fraction of fertilized females. Thereby, sexual selection reduces the females mating success and causes fertilization limitation. Sexual selection can increase the fitness of the individuals; however it may also have a negative effect at the population level in terms of reduced population growth rate.

Chapter 3: Age- and size-dependent mating performance and fertility in a pelagic copepod, Temora longicornis

In this study we examined age- and size-dependent reproductive performance (egg and sperm production, mating success) in *Temora longicornis*. We modified existing DAPI protocols used for insects, bacteria and blue-green algae to stain the spermatozoa so we could count the number of spermatozoa inside the spermatophore. Our results showed 3 clear findings. *i*) Ageing effects were evident. Young males had a higher mating success and a higher reproductive success compared with old males. Mortality rate increased and fertility decreased rapidly with age. The latter effect was stronger in males than females. Also, in both genders, the reproductive period was significantly shorter than the average longevity. Under optimal laboratory conditions the average adult longevity was 30 d in both males and females, but females produced eggs for only 18 d, and males could fertilize females for only about 8 d after they matured. *ii*) Several aspects of reproductive performance increased with size in both males and females. We found that larger males, compared with smaller males produced larger spermatophores containing more spermatozoa and fertilized a larger fraction of available females. We also found that females mating with large males produced more offspring than those mating with small males. Similarly, large females had higher egg production rates as well as a higher lifetime egg production than did small females. *iii*) Repeated mating was not only potentially advantageous (e.g. in terms of higher genetic variability) for females, but came at the expense of higher mortality.

We propose that the strong size- and age-dependent fertility observed in this species is conducive to the evolution of sexual selection via mate choice for young and large partners.

Chapter 4: Identification and characterization of six microsatellites primers for the calanoid copepod Temora longicornis

Many marine organisms are difficult to observe while mating, and it is usually impossible to determine which individuals are successful (Karl 2008). This applies to copepods as well, and previous experiments to examine mating in copepods have been designed as one-male-to-one female incubations or as incubations where the successful males cannot be identified.

This technical note describes the identification and characterization of the microsatellite primers used to do paternity analysis in Chapter 5. Microsatellites are short tandem repeats of 1-6 nucleotides and alleles are distinguished by varying number of repeat units (i.e. total length). Microsatellites are generally believed to be positioned in the non-coding region of the chromosome and they are expected to be under neutral selection. Due to their Mendelian inheritance the allele frequencies are expected to be in Hardy-Weinberg

equilibrium. Microsatellites may allow paternity assignment of animals, and recently microsatellite loci have been developed for several species of copepods (Harrison et al. 2004, Zeller & Reusch 2004, Todd et al. 2004, Provan et al. 2007, Ferrada et al. 2011). As adult copepods and early life stages (eggs and nauplii) contains very little tissue, we tested four different DNA extractions methods (E.Z.N.A® Tissue DNA Kits (Omega Bio-Tek), NucleoSpin-Tissue-Kit (Macherey-Nagel), Wizard Genomic DNA Purification System kit (Promega) and Chelex-100 resin (BioRad)) and we found the greatest success in PCR amplification for samples extracted by Chelex-100 resin. We used "*454 next generation sequencing*" (NGS) to identify and characterize microsatellites for *T. longicornis*. A total of 12,206,429 bases with 30,851 sequences were obtained with an average sequence length of 395 bases. We tested 17 microsatellites primer pairs, 11 were discarded either due to low levels of polymorphism, insufficient information content or difficulty too amplify or score, leaving a total of six informative and consistently amplified loci. The loci found in this study fulfil the quality criteria, i.e. high levels genetic variability, independence of loci and no non-amplifying alleles or PCR artefact, for parentage assignment.

Chapter 5: Mating success and sexual selection in a pelagic copepod, Temora longicornis: Evidence from paternity analyses.

This study was designed to follow up on the findings from Chapter 2 and 3. To investigate if mating was random or under the influence of sexual selection, we used the microsatellites primers developed in Chapter 4 to do paternity analysis of the female's offspring. Marine organisms are difficult to observe during the mating, and it is often impossible to determine which males are successful (Karl 2008). However by establishing paternity, we can precisely quantify the reproductive success of individual males and thus assess how successful their particular reproductive strategies have been. The aim of this study was to examine *i*) the occurrence of multiple paternity in *Temora longicornis ii*) the effect of multiple paternity (if present) on the females reproductive output, and *iii*) whether mating is random or some individuals have a higher-than-average chance of fertilizing or being fertilized ('super individuals').

Our study clearly demonstrated the occurrence of multiple paternity in *Temora longicornis*. This is the first time in which genetic polyandry has been documented in a pelagic copepod. Multiple paternity opens up for the possibility of post-copulatory sexual selection, such as sperm competition and cryptic female choice. In this study we found elevated offspring production in females that mated repeatedly. This increase in offspring production is unlikely to be caused by a more plentiful sperm supply because one spermatophore contains sufficient sperm to fertilize all the eggs that a female produces in her life-time (Chapter 3). Our observation rather suggests that post-copulatory sexual selection increases the reproductive output in females with multiple paternities.

In accordance with the findings in Chapter 2, we found that a substantial fraction of the males (8-14 %) in our study never mated during incubations, despite a plentiful supply of females. We also found that only about half of the females in our experiments were fertilized, even though male availability was high and mate encounters not limiting. From a population level it is undesirable to have such a high fraction of unfertilized females and unsuccessful males.

This study represents a new approach and one of the first steps towards a more comprehensive understanding of mating systems in copepods. We suggest that sexual selection in both sexes through (cryptic) mate choice and/or sperm competition may account for the skewed mating success of both males and females. We further found that mating was non-random. Superior individuals with a higher than average mating success were identified both among females and among males. Some of the variation between individuals could be explained by variation in size and age.

8. Overall conclusion

The findings of this PhD thesis provided evidence that sexual selection exists in *Temora longicornis* and that it may operate via mate choice, with both males and females preferring to mate with large and young partners. We have shown that in both males and females, mating was non-random. Superior individuals with a higher than average mating success were identified both among females and among males.

We have shown that multiple mating can lead to multiple paternity. This is the first time that genetic polyandry has been documented in a pelagic copepod. This also opens up the possibility that post-copulatory sexual selection occurs in copepods, such as sperm competition and cryptic female choice.

We have found that a substantial fraction of the males both in the field and in the lab populations never mated during incubations, despite having a plentiful supply of unfertilized females. This indicates that a fraction of the male population is excluded from the reproductive pool and thus not contributing to the future population.

The fact that males can mate with more than one female is one of the basics for sexual selection. Thus it may be even more surprising that we found such a high fraction of males there did not mate successfully, despite a high availability of females.

9. Future perspectives

Sexual selection is a fundamental force driving the evolution of behavioural, anatomical, and physiological traits in nearly all sexually reproducing organisms. Unlike traits that merely offer a survival advantage, sexually selected traits are those that directly enhance an individual's relative reproductive fitness. For a better understanding of evolutionary and ecological processes, we need to gain more knowledge about the mechanisms and impact of sexual selection.

Sexual reproduction comes with a trade-off between benefits and costs. If these can be quantified we may predict the optimal strategies. This knowledge can be used to expand existing models of optimal swimming strategies to include competition for mates and reduction of mating rates due to prolonged mate choice. This could lead to improved predictions of optimal motility behaviours, the related mortality risks, and the consequences for population dynamics.

Copepods are a major food source of numerous species of fish larvae. In particular, they serve as primary prey for early life history stages of many fish species of economic importance. Fish harvested from aquaculture is predicted to make up 50% of the world's seafood supply (Sofia 2010). However, due to difficulties in rearing sufficient quantities of copepods and the high associated costs, copepods are still not widely used in the aquaculture industry. Therefore, determining the factors that govern copepod productivity is essential. Further development and optimization of the production of live feed for fish production is needed and knowledge about the factors controlling mating is essential to secure a high and cost-efficient production of copepods.

Due to the ecological importance of copepods in aquatic ecosystems, it is critical to understand their population dynamics. Since the future evolution of copepods in a changing environment is determined by differential lifetime fitness of individuals, we need to understand reproductive behaviours in order to predict their genetic diversity and adaptive abilities to novel conditions. This PhD study represents a new approach and one of the first steps towards a more comprehensive understanding of mating systems in copepods. These first results are promising; however, there is a need to expand this type of analysis to other copepod species and to explore the generality of our results in other mating scenarios and also for other planktonic organisms.

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CHAPTER 2

Low fertilization rates in a pelagic copepod caused by sexual selection?

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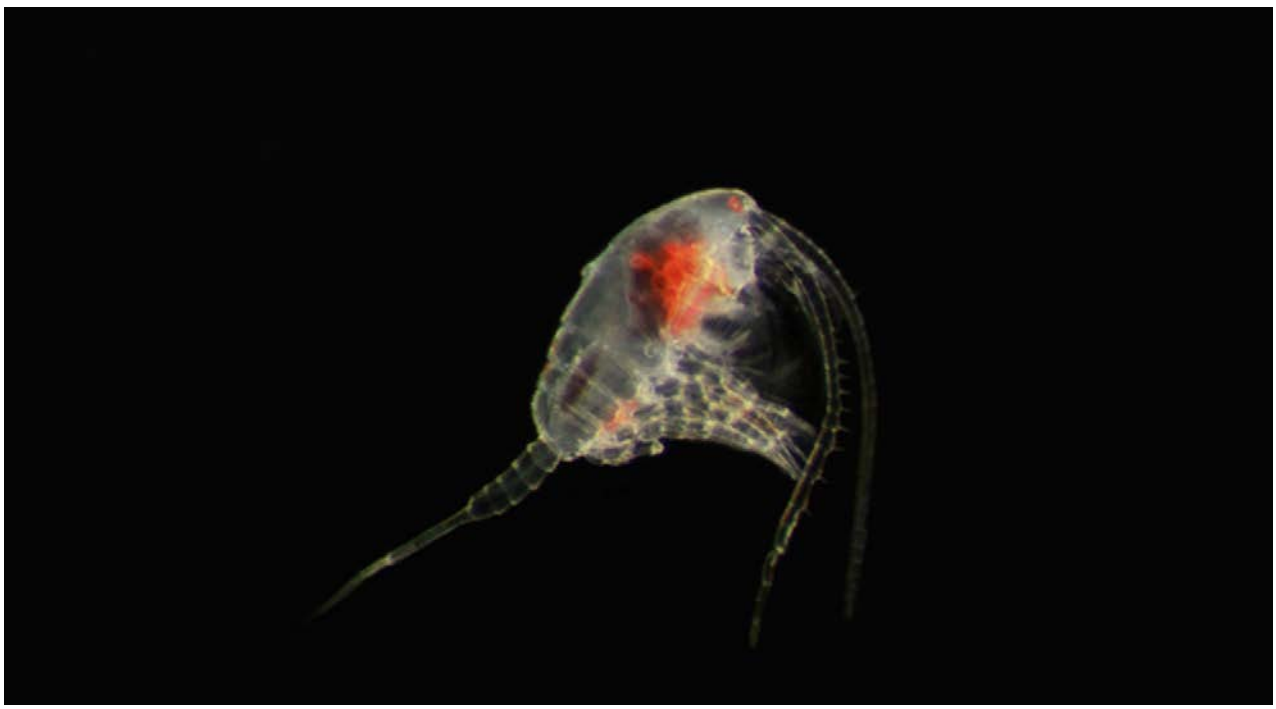

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Low fertilization rates in a pelagic copepod caused by sexual selection?

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We studied female fertilization status in North Sea summer populations and laboratory cultures of the marine copepod *Temora longicornis* and found fractions of fertilized females in both field and laboratory populations that were much smaller (<50%) than predicted by a theoretical model that assumes random mating. Such low fertilization rates are normally related to environmental factors such as poor food or low densities, which we could not confirm in our experiment. Male density was negatively related to fertilization rate, and a large fraction of males did not mate in laboratory incubations. We therefore suggest that sexual selection, through mate choice or male–male competition could account for low fertilization rates of females in populations of pelagic copepods during some periods of the year.

KEYWORDS: fertilization limitation; male–male competition; mate choice; mating rates; zooplankton

INTRODUCTION

Female sperm limitation seems to be common in marine benthic invertebrates with external fertilization, where it affects population dynamics and life-history strategies (e.g. Levitan and Petersen, 1995). Most pelagic invertebrates have internal fertilization, and sperm limitation may similarly occur in, for example, copepods (Parrish and Wilson, 1978; Kiørboe, 2007). However, observations of the fraction of fertilized females in copepods or other zooplankton field populations are rare (but see Hopkins, 1982; Uye and Sano, 1995). Low egg-hatching rates in field and lab populations of copepods have been attributed to detrimental or poor female diets (e.g. Ianora *et al.*, 2003; Jónasdóttir *et al.*, 2009). Males have been

almost neglected in studies of the reproductive ecology of copepods, and the limited knowledge about male reproduction and mating ecology comes from few laboratory studies that show low male mating rates (Kiørboe, 2007; Ceballos and Kiørboe, 2010, 2011; Sichlau and Kiørboe, 2011). Poor male mating performance could strongly limit female fertilization even when population abundance is high enough to ensure a high mate encounter rate (Kiørboe, 2006, 2007) and could explain cases of low egg hatching success under otherwise suitable environmental conditions. However, the effect of male abundance on female fertilization status has not been studied in natural populations of pelagic copepods.

Sexual selection offers alternative explanations to low fertilization rates, because mate competition, mate choice and mate coercion can decrease mating rates (Andersson, 1994), and hence could lead to reduced population viability (Møller and Legendre, 2001; Kokko and Brooks, 2003). Recent empirical and theoretical studies have demonstrated sexual selection by mate choice in copepods (Ceballos and Kiørboe, 2010, 2011; Sichlau and Kiørboe, 2011; Heuschele *et al.*, 2012). The strength and direction of sexual selection is affected by mate availability and encounter rates (Kokko and Rankin, 2006), but this has not yet been experimentally addressed in copepods.

Here, we examine the fertilization status of the pelagic copepod *Temora longicornis* from laboratory and North Sea summer populations, and compare them to theoretical predictions that assume non-selective matings. We quantify male mating rates and examine whether the fertilization of females is influenced by adult abundance, male mating capacity and the environment, and discuss the potential role of sexual selection.

METHOD

Sampling and culturing

We performed experiments with wild and lab cultured copepods. The field study was carried out during a cruise in the North Sea in August 2010 (Fig. 1). We sampled six stations between 5:00 and 6:00 AM (local time). Adult copepods were collected by several vertical net hauls from 1 m above the bottom to the surface, using a WP-2 plankton net (250-μm mesh size). The contents of one net haul per station were preserved in 4% formaldehyde in seawater to analyze density and sex ratio. We recorded water column profiles of temperature, salinity and fluorescence using a CTD (Seabird SBE 911+) attached to a rosette sampler equipped with 12 Niskin bottles of 5 L each. Phytoplankton biomass was estimated as the Chlorophyll *a* (Chl-*a*) pigment concentration in the

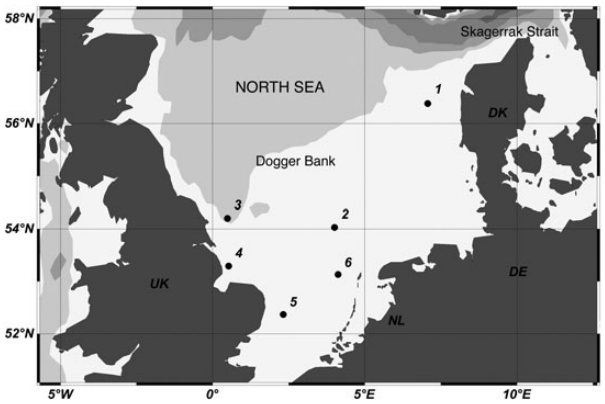

Fig. 1. Study area showing location of sampled stations.

maximum fluorescence depth and at the surface. The protocols for pigment extraction in ethanol and concentration measurements were standard (Jespersen and Christoffersen, 1987). Water for incubation experiments was collected with Niskin bottles at the depth of the fluorescence maximum. If no distinct maximum fluorescence layer was present, we collected water from 10 to 20 m depth. This water was passed through a 40 μm mesh to remove mesozooplankton, eggs and nauplii before being used in the experiments.

Our lab culture came from copepods originally isolated from the Kattegat area (Baltic Sea), and they were kept in continuous cultures in darkness, at 14°C and a salinity of 32 PSU in the laboratory at The National Institute of Aquatic Resources in Charlottenlund (Denmark). The cultures were fed a mixture of *Rhodomonas salina*, *Prorocentrum minimum*, *Thalassiosira weissflogii* and *Heterocapsa triquetra*.

Experiments

All experiments were run at 14°C in a cold room with 12/12 h dark/light period onboard the research vessel DANA or in darkness in the laboratory. Incubation bottles were sealed without a head space and rotated on a plankton wheel (1 rpm) to avoid the sedimentation of food particles. Rotation does not affect mating success in copepods (Choi and Kimmerer, 2009). In the laboratory, copepods were fed a mixture of the flagellate *R. salina* and dinoflagellate *P. minimum* provided in excess (>300 μg C L^{-1}, Dam and Peterson, 1991). At sea copepods were fed on the natural food assemblage, as we were interested in *in situ* data.

Fraction of fertilized females

We measured the fraction of female *T. longicornis* that produced fertilized eggs (fraction of fertilized females: FFF) in field and in laboratory populations, by individually

incubating females in 69 mL flasks. After 36 h females were removed and the flasks checked for eggs. We considered a female as fertilized if we found at least one nauplius after additional 60 h incubation. The number of replicates (22–57) per station varied with availability of copepods. In the laboratory, we incubated 30 females from each of four culture tanks. Each culture tank is an independent population that differed in age distribution and thus population composition.

To test whether fertilization rates are driven by sex ratio and density alone, we compare our observed fraction of fertilized females with a theoretical fraction derived from a model developed by Kiørboe (Kiørboe, 2006). This steady state model is based on the calculation of mate encounter rates and assumes no mate selectivity and that each encounter leads to a successful mating. An encounter is defined as the male finding the pheromone signal of a female. In *T. longicornis* males are searching for females. The model applies to species where re-insemination is needed, because *T. longicornis* lacks a spermatheca, and males have a limited mating capacity. It is formulated as follows:

$$\mathrm{FFF} = 1 - \exp[(-\beta\, C\, \pi\, t)/(\pi + 1\, \alpha\, \beta\, C)]$$

The mate encounter rate depends on the product of the volumetric mate searching rate, β (i.e. the volume of water males can screen daily for females; 0.17 m^3 day^{-1} from Doall *et al.*, 1998), on the concentration of adults C (ind. m^{-3}), and on the sex-ratio π (males/females). The number of matings is reduced by a male reproductive 'time out', α (i.e. time needed to produce a new spermatophore as estimated in our experiments). The model also accounts for the reproductive 'time out' of the female, t, i.e. the duration of time after one mating that the female needs no re-mating to fertilize eggs; 13.56 days (own results).

Male mating rate

To measure maximum male mating rate, we incubated 15 groups, each consisting of 1 male and 10 females of field-caught *T. longicornis*, for 24 h in 700 mL bottles. This incubation volume does not limit mate-searching rate in small copepods (Kiørboe and Bagøien, 2005; Choi and Kimmerer, 2009), and the male mating rate was not encounter limited due to the high availability of females, which also minimizes the potential effects of mate selection. We used only females without attached spermatophores to increase their willingness to mate during the incubation. To capture the natural variability related with mating history, males were not isolated prior to the experiment. After the incubation, we counted the number of spermatophores, lost or attached to females. Mating rate is equal to spermatophore production rate because males transfer one spermatophore per mating event. We also measured male mating rate in laboratory cultures using 24 h old virgin adults to minimize effects of non-receptive females and individual's age on mating rates (Ceballos and Kiørboe, 2011). Forty-five couples were incubated during 24 h in 69 mL flasks (Choi and Kimmerer, 2009), and fed *ad libitum*. There was no difference between field and lab mating rates (see below) and we consider the potential bias due to different methodology negligible. We calculated the male reproductive 'time out', i.e. the time males need to produce a new spermatophore, as the inverse of the male mating rate.

Statistical analysis

When analyzing the influence of environmental parameters on the fraction of fertilized females, we used a generalized linear model (GLM, McCullagh and Nelder, 1989) with binomial distribution with individual fertilization status (1/0) as the dependent variable, and included chlorophyll *a* concentration in the maximum fluorescence depth, mating rate of males, average temperature, adult density and sex ratio as independent factors in the starting model. We then reduced the model to the minimum adequate model using stepwise backward elimination based on statistical significance level. To investigate further the effect of male and female density, we used a similar GLM but with male and female density as independent factors. The difference in male mating rate among stations was analyzed using a GLM assuming a quasi-poisson family (due to the overdispersed count data, Zeileis *et al.*, 2008). The number of matings per male was used as dependent variable and station as fixed factor. We compared this model and one without station by using a Chi-square test. We compared the mating rates from the field and the laboratory using a GLM assuming a quasi-poisson distribution, with location (laboratory/field) as the independent factor and mating rate as dependent factor. We used the Chi-square test to compare the frequency of fertilized females among stations, and data were checked for homogeneity of variance with the Bartlett's test. The analyses were run using R (R Core Team, 2013).

RESULTS

Surface temperature varied between 14 and 18°C during the survey and phytoplankton biomass was quite high given the time of the year (Fig. 2A and B, Richardson *et al.*, 1998). Densities of *T. longicornis* ranged from 19 to 101 individuals m^{-3} and sex ratios (males/females) were around 1 at most stations (Fig. 2C).

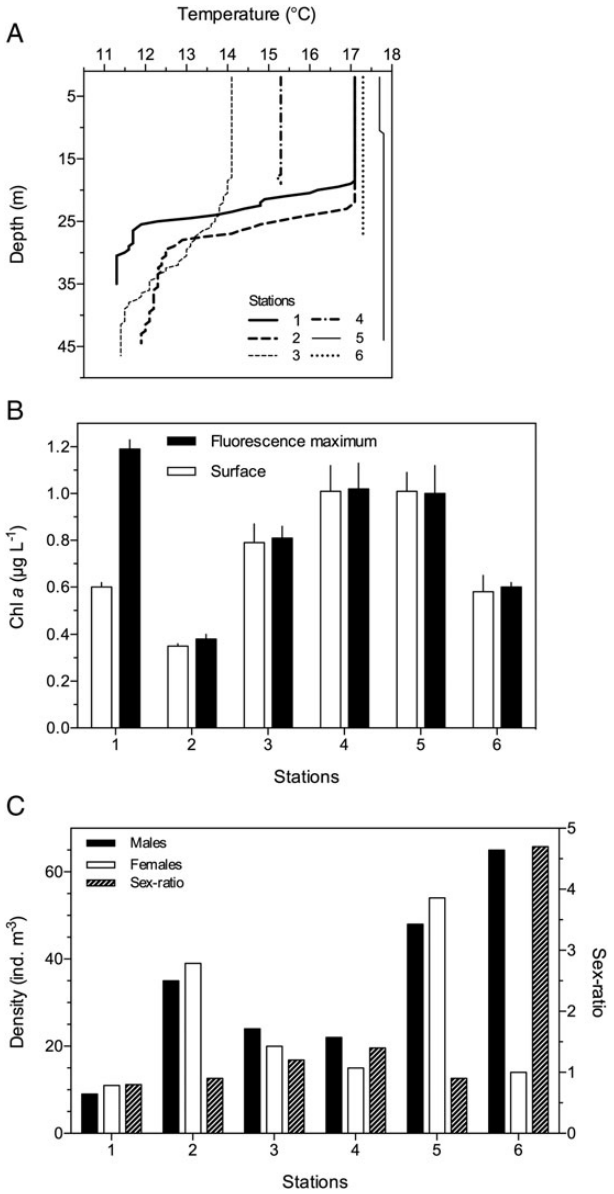

Fig. 2. Environmental conditions and population parameters in the field. (**A**) Vertical profiles of temperature. (**B**) Chlorophyll *a* (Chl *a*) concentration, which is shown as mean ± 1 SD, $n = 3$. (**C**) Density of males and females, and sex-ratio.

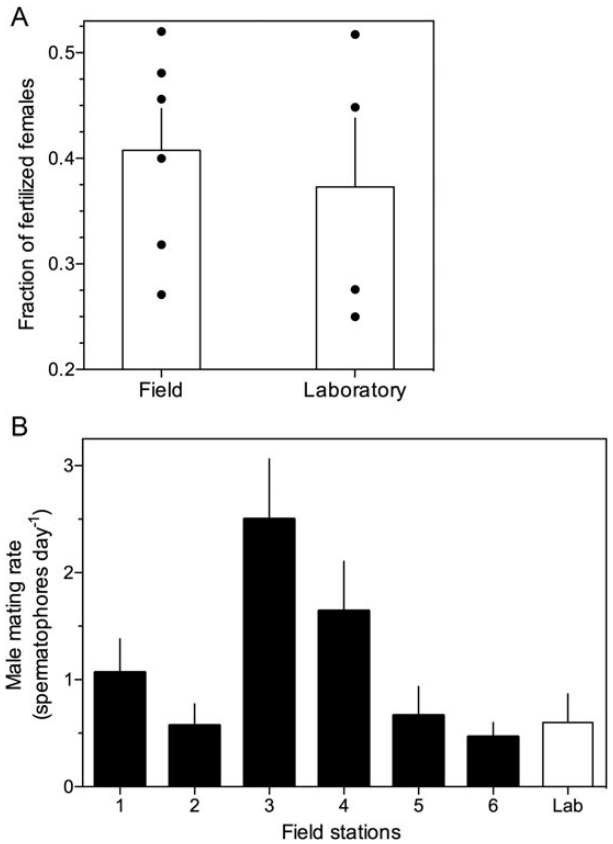

Fig. 3. (**A**) Fraction of fertilized females (FFF) in field populations and laboratory cultures. Single data are shown as points and columns represent mean ± 1 SE. The theoretical values of FFF predicted by the model are 1 for all stations and laboratory cultures. The empirical FFF did not vary significantly among stations (Chi-square test: $\chi^2_5 = 0.20$, $P = 0.999$). (**B**) Male mating rates in the field and in the laboratory (mean ± 1 SE). Lab-cultured and wild males showed the same mating capacity (GLM: estimate = 0.129, SE = 0.177, $t = -1.67$, $P = 0.098$), but male mating rate varied for the different field stations ($\chi^2_5 = 35.7$, $P < 0.01$).

On average 41% (range: 27–52%) of the field females were fertilized (Fig. 3A). In the dense laboratory cultures (10 000–31 000 individuals m^{-3}), the FFF was similarly low (average: 37%, range: 25–52%). The theoretical model predicted that all the females should be fertilized when using the measured population parameters as input. The overestimation ranged between 48–73% and 48–75% for field and lab populations, respectively. To make predictions and observations match, the male search volume would need to be two to three orders of magnitude lower than what is observed in this species (Doall *et al.*, 1998). This suggests that only between 0.1 and 1% of the male–female encounters result in successful mating.

Neither temperature, food availability, sex ratio, nor male mating rate explained the variance in the FFF (Table I), but the FFF was negatively correlated with both adult and male density (Table I).

On average, males mated <1.5 times per day and a large fraction (28–57%) of the males did not mate at all. Male mating rate varied significantly among stations, from 0.5 to 2.5 matings day^{-1} (Fig. 3B). Maximum individual mating rate was 8 day^{-1}, and in the laboratory some males mated up to 10 times per day (Fig. 3B). Male 'time out' of reproduction in the field varied for the different stations (Fig. 3B) and averaged 17.9 ± 3.7 h (SD). The shortest 'time out' was just 2.4 h and found in the

Table I: Influence of environmental and population parameters on the fraction of fertilized females

All variables					Sex specific densities				
	Estimate	SE	Z-value	*P*-value		Estimate	SE	Z-value	*P*-value
Intercept	0.25	0.27	0.90	0.368	Intercept	0.20	0.26	0.76	0.447
Adult density	−0.01	0.00	−2.30	0.021	Male density	−0.02	0.01	−2.23	0.026
Sex ratio	−0.07	0.13	−0.56	0.576	Female density	−0.01	0.01	−0.67	0.503
Phytoplankton biomass	−0.53	0.50	−1.07	0.286					
Temperature	0.17	0.18	0.93	0.352					
Male mating rate	0.61	0.60	1.01	0.315					

Summary of generalized linear models.

laboratory. The male mating rate was not influenced by the sex ratio, or by female, male or adult abundance in their original population, and it was similar between the laboratory and the field (Fig. 3B).

DISCUSSION

We found a low fraction of *T. longicornis* fertilized females in North Sea summer populations and in laboratory cultures even when male availability was high and mating not encounter limited. At low population density, sperm limitation and 'Allee effects' may affect copepod population dynamics (Choi and Kimmerer, 2009). Sperm or mating limitation has only rarely been considered as an important factor for copepod population dynamics (Kiørboe, 2007; Choi and Kimmerer, 2008, 2009), and our theoretical calculation suggests that mating limitation was insignificant in both lab and field populations. Although there is plenty of information about egg hatching success for many species, including *T. longicornis* (e.g. Maps *et al.*, 2005; Dutz *et al.*, 2012), published data on fertilized frequency are rare (Hopkins, 1982; Uye and Sano, 1995). Why are such a low fraction of females fertilized?

There is the possibility that we might have mistaken females that produced resting eggs for unfertilized females in the field, but we think that this is unlikely to be a significant source of error because (i) the production of resting eggs in *T. longicornis* peaks in spring and early summer (Castellani and Lucas, 2003; Engel and Hirche, 2004) and (ii) we would count a female as fertilized even if only 1 of the eggs hatches. Given hatching rates during August of ∼20–40% (Castellani and Lucas, 2003), we are convinced that our results should not be significantly biased by this possibility. Additionally, post-reproductive females may account for the occurrence of non-fertilized females as *T. longicornis* is known to survive beyond its reproductive time period (Sichlau and Kiørboe, 2011). We assessed the fraction of post-reproductive females by assuming a constant adult mortality rate, δ, and a duration of the reproductive period (from maturation to cessation of producing fertilized eggs), $\mathcal{T}$. The fraction of post-reproductive females in a population can then be estimated as:

$$\int_{\mathcal{T}}^{\infty} \mathrm{e}^{-\delta t}\mathrm{d}t / \int_{0}^{\infty} \mathrm{e}^{-\delta t}\mathrm{d}t = e^{-\delta \mathcal{T}}.$$

With $\mathcal{T} = 18$ days (Sichlau and Kiørboe, 2011) and $\delta = 0.1$ days^{-1} (Hirst and Kiørboe, 2002) post-reproductive females constitute at most 16% of the females, which is much less than the observed fraction of unfertilized females. Moreover, a mortality of 0.1 day^{-1} is typical for small copepods, but it may be higher in August when predation pressure on copepods is at its annual maximum in the North Sea (Kiørboe and Nielsen, 1994). Therefore, our calculation may even overestimate the fraction of post-reproductive females.

Food quality, sex-change and temperature may explain low hatching rates in copepods and low percentages of spawning females (e.g. Maps *et al.*, 2005; Gusmão and McKinnon, 2009; Jónasdóttir *et al.*, 2009, Dutz *et al.*, 2012), but this seems not to be the case in our study. Even in the lab cultures, where mate encounter rate and food quality are optimal and egg hatching success is near 100% (Jónasdóttir and Kiørboe, 1996), a large fraction of the females were not fertilized. Seasonality is unlikely to explain the low FFF mainly because there is no seasonality in the lab cultures, yet we find many unfertilized females.

Limited male mating capacity can explain fertilization limitation in populations of the copepod *Oithona davisae* with a very female-biased sex ratio and low male spermatophore production (Kiørboe, 2007). However, this mechanism cannot explain the low fertilization fraction in female *T. longicornis*, because we observed a decrease in this fraction with increased population and male density. Additionally, the 'time out' of reproduction for *T. longicornis* males is short enough to ensure high numbers of fertilized females. It is striking that >25% of the males did not mate despite a plentiful supply of females.

Our results suggest that mating in *T. longicornis* is not random and accordingly the model assuming no mate choice clearly overestimates FFF. A male may encounter a female signal without this leading to a successful mating and there can be several explanations for this. The male may lose the female trail before encountering the female (Bagøien and Kiørboe, 2005). Predation risk can reduce sexual activity and mating success in copepods (Lasley-Rasher and Yen, 2012), but predation does not explain our results because FFF was also low in our lab populations. A mating is only successful if the female gets fertilized, which requires the male to correctly transfer and attach the spermatophore in the female's genital opening. Wrong placements of spermatophores have been described before (Blades, 1977; Hopkins and Machin, 1977), and the loss of the spermatophore during the transfer is also possible. However, such mistakes are unlikely to be common in such a fitness related trait, and therefore it is unlikely to explain the high percentage of non-fertilized females and the computed low success of encounters that we found.

We propose that sexual selection by mate choice, male–male competition and other reproductive behaviors could affect the mating activity of copepods, reduce female mating success and cause fertilization limitation. For example, in *T. longicornis* males use female pheromones to locate females (Doall *et al.*, 1998), and males that have found a trail may disrupt it and prevent further males from finding this female (Uchima and Murano, 1988). Thus, the males with the best female-searching skill would be favored in a context of male–male competition. Our proposal is consistent with recent experimental and theoretical studies that have demonstrated sexual selection by mate choice in three copepod species (Ceballos and Kiørboe, 2010, 2011; Sichlau and Kiørboe, 2011; Heuschele *et al.*, 2012).

This study is the first to present field observations that suggests significant effects of sexual selection on population dynamics in zooplankton. How these effects drive mating behavior, their relative importance in different populations and through the seasonal cycle is unknown. Sexual selection can speed up adaptations to changing environments and may have contributed to the evolutionary success of copepods in the oceans. However, it may also have negative impacts at the population level in terms of reduced population growth rate (e.g. Møller and Legendre, 2001). The topic deserves further consideration in future studies of zooplankton mating behavior and population dynamics.

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CHAPTER 3

Age- and size-dependent mating performance and fertility in a pelagic copepod, *Temora longicornis*

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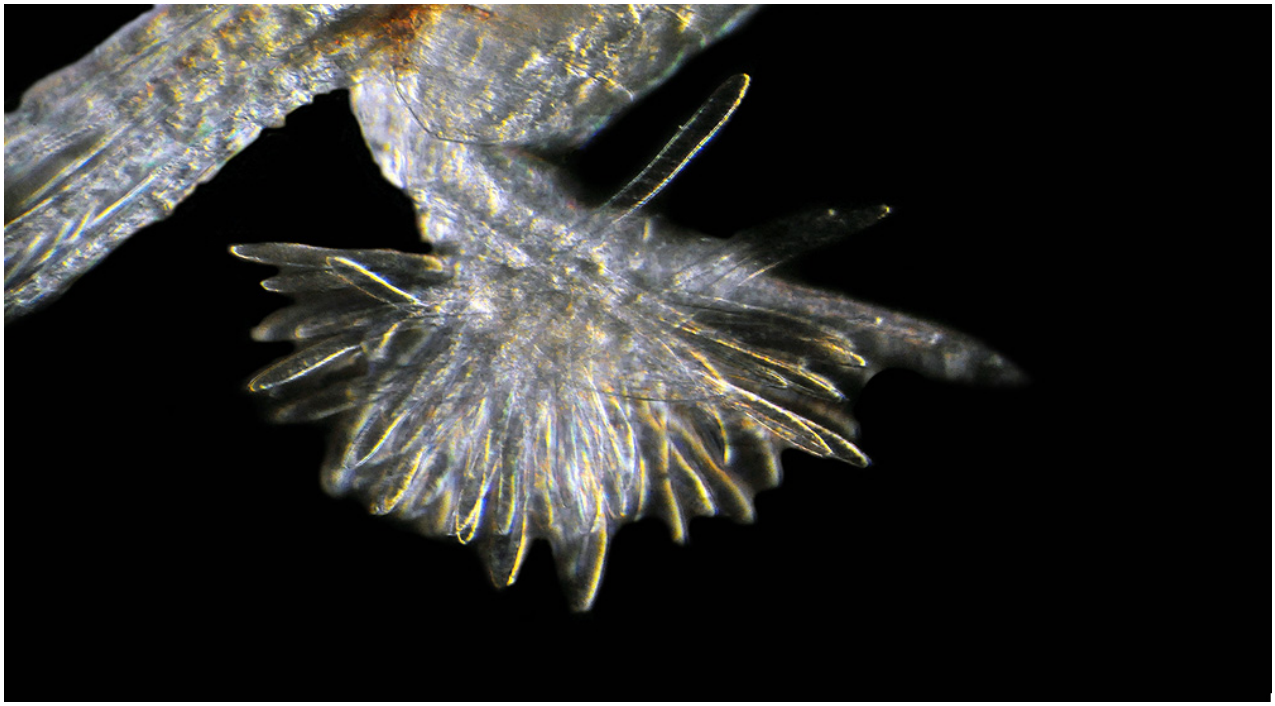

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ABSTRACT: In many species, size and age can be strong determinants of the reproductive success for both sexes. We examined age- and size-dependent reproductive performance (egg and sperm production, mating success) in a pelagic copepod, *Temora longicornis*. Compared with smaller males, larger males produced larger spermatophores containing more spermatozoa and fertilized a larger fraction of available females. Females mating with large males produced more offspring than those mating with small males. Similarly, large females had higher egg production rates as well as a higher lifetime egg production than did small females. Ageing effects were evident in this species: mortality rate increased and fertility decreased rapidly with age. The average adult longevity under optimal laboratory conditions was 30 d in both males and females, but females produced eggs for only 18 d, and males could fertilize females for only about 8 d after they matured. The strong size- and age-dependent fertility observed in this species is conducive to the development of sexual selection via mate choice for young and large partners, as has been shown in another copepod species.

KEY WORDS: Spermatophore · Spermatozoa · Egg production · Ageing · Mortality rate · DAPI · *Temora longicornis*

INTRODUCTION

Reproduction and mate choice are important determinants of the evolution and life history of all organisms (reviewed in Andersson 1994), and in many species both depend on the body size and age of the individual. Generally, larger individuals of a species produce more gametes and their mating performance typically declines with age (MacDiarmid & Butler 1999, Radwan et al. 2005, Lehmann & Lehmann 2009, Gasparini et al. 2010, Judge et al. 2010). Here we examined how reproductive performance and mortality depended on size and age in a pelagic copepod, *Temora longicornis*.

Numerous studies have shown that the variation in copepod fecundity is related to changes in food quantity and quality (Jonasdottir et al. 1995, 2009, Dam & Lopes 2003, Koski et al. 2006), salinity (Holste et al. 2009) and temperature (Ban 1994, Holste et al. 2009), but few have tested the effect of ageing on fecundity (Carlotti et al. 1997, Rodriguez-Grana et al. 2010, Ceballos & Kiørboe 2011). Ageing is the progressive decline in biological functions with advancing age and the accumulation of change in an organism over time. Ageing can have a profound negative effect on the individual fitness of some pelagic copepod species, e.g. on feeding rates, oxidative damage, egg production rate and egg hatching success (Carlotti et al. 1997, Rodriguez-Grana et al. 2010, Ceballos & Kiørboe 2011). Male ageing can have evolutionary and ecological consequences on gamete performance and fitness (see Pizzari et al. 2008). The quality of the genes that a male passes to his progeny may change with his age (Hansen & Price 1995) owing to the accumulation of deleterious mutations in the germline (Risch et al. 1987, Drost & Lee 1995,

Hurst & Ellegren 1998, Bartosch-Härlid et al. 2003, Glaser & Jabs 2004). Ageing can also affect the quantity of sperm because an increasing proportion of germ cells bear mutations as age increases, and such cells are destroyed by genetic self-guarding processes (Radwan 2003). Older males, however, have passed more episodes of selection and thus may be better adapted than the average male (Manning 1985). The breeding value (i.e. offspring performance) of older males will thus depend on specific life history characteristics (Kokko 1998, Beck & Powell 2000) and on the number of divisions a male germline undergoes after it reaches maturity (Radwan 2003). Therefore, it is not surprising that female preferences with respect to the age of the male differ among species (reviewed in Brooks & Kemp 2001). Little is known about the reproductive performance of male copepods, and less about the age dependency of male reproduction.

Adult body length of many copepods may vary substantially with both temperature and food availability by up to a factor of 2 within a species (Berggreen et al. 1988, Dam & Peterson 1991, Arendt et al. 2005). Female fecundity typically increases with body size in both insects and crustaceans, including copepods (Honêk 1993, Aquiloni & Gherardi 2008, Ceballos & Kiørboe 2010). Spermatophore size and sperm quantity is similarly correlated with male size in insects and a single copulation by a small mature male may not provide enough sperm to fertilize all the eggs produced by a large female (McLain et al. 1990, Bissoondath & Wiklund 1996). Ceballos & Kiørboe (2010) showed that large males of *Acartia tonsa* produce larger spermatophores than do small males, and that the production of offspring in the female increases with the size of the spermatophores she receives and thus with the size of the male, probably owing to a larger content of spermatozoa in larger spermatophores. Because sperm are small and typically produced in very large numbers compared with eggs, sperm are traditionally considered an unlimited resource, but such observations suggest that sperm can be limiting. Dewsbury (1982) pointed out that sperm delivered to females in ejaculates or spermatophores, as in copepods, may be costly or slow to produce, and there is growing evidence to indicate that sperm supply can limit fertilization success and realized fecundity (Wedell et al. 2002). Spermatophore production may involve significant energetic investments for male copepods (Mauchline 1998), but the contents of sperm and semen in spermatophores is unknown in copepods. Copepod spermatophores contain various substances besides spermatozoa (Defaye et al. 2000); if such substances are nutritional, large spermatophores may increase the female's fecundity, as found in other taxa such as insects. Female copepods may therefore have an advantage in mating with large young males, because (1) it reduces her need for frequent mating and thus reduces the potential risks of mating and (2) she receives sperm of high quality and quantity, which gives her a higher reproductive success. In this study we tested the hypothesis that size and age are significant factors influencing male fecundity (mating rate and sperm production) and reproductive fitness in the pelagic copepod, *Temora longicornis.*

MATERIALS AND METHODS

Experimental copepods and general procedures

Experiments were conducted with the calanoid copepod *Temora longicornis*, which was originally obtained from the central North Sea but has been cultured in our laboratory for over a year. Cultured *T. longicornis* decrease in size during subsequent generations (Klein-Breteler & Gonzalez 1982). The culture was maintained and all experiments were conducted with food provided in excess, in darkness, at 14°C and at a salinity of 32. Temperature and salinity were within the range in which the species thrives optimally (Maps et al. 2005). *T. longicornis* is a broadcast spawner and both the female and male engage in multiple matings. The adults measure about 1 mm in cephalothorax length. Females have normally 1 to 3 attached spermatophores. In this paper, 'age' refers to age since maturation; this is closely related to absolute age because maturation age varies very little for individuals grown under standardized conditions.

Most experiments (Expts 1 to 4) were conducted with virgin adults. To make sure that the copepods were virgins, late copepodites (Stages CIV to CV) were incubated individually in 69 ml bottles. The bottles were inspected daily to obtain freshly moulted virgin adults. Copepods were incubated with food provided in excess (1000 µg C l^{-1}, phytoplankton of species *Rhodomonas salina* and *Prorocentrum minimum*). Copepods in Expt 1 were incubated in 69 ml bottles and those in Expts 2 to 4 were incubated in 630 ml screw-cap bottles. All bottles were sealed without a head space and placed on a plankton wheel that rotated at 1 rpm.

Expt 1: longevity of adult females and males

Four males or females that had matured within 24 h were placed in each experimental bottle, 75 bottles for each sex. Every second day the numbers of live copepods were counted, dead ones were removed, and new food and water was added. The experiment continued until all copepods had died. The average duration of adult life was computed as the averages of the individual death dates (with t_0 = maturation date).

Expt 2: duration of the fertile period of females, lifetime egg production and age-dependent egg production rate

One virgin female and one virgin male were placed in each experimental bottle (N = 10). After 24 h, the female was isolated and her egg production monitored daily until she did not produce eggs for 4 consecutive days. Every day the female was transferred to a new bottle, the remaining water was filtered and the eggs were counted. The experiment was repeated using a slightly modified design to ensure that the females were unlimited by mating opportunities and sperm: The virgin females (N = 10) were offered 3 males (to prevent mating and sperm limitation) and the males were replaced every 48 h throughout the experiment. Female egg production was monitored as above.

Expt 3: age- and size-dependent male mating performance and fertility

To estimate mating performance of males (quantified as the fraction of mating opportunities actually used) as a function of their age and size, 1 virgin male and 2 virgin females were placed in each of the 10 experimental bottles. Every 24 h the females were replaced with 2 new virgin females that had matured within 24 h. The old females were transferred individually to a new experimental bottle containing seawater and food in excess, and their nauplii production was followed daily for 10 d to determine whether the female had been fertilized and to estimate the effect of male size on the total number of nauplii that a female could produce after 1 mating. Each male was offered a total of 20 females over a period of 10 d. The size of the male was measured under a dissecting microscope.

Expt 4: size of the spermatophore

To examine the size of the spermatophore relative to the size of the male, virgin couples were incubated for 24 h (N = 115). Each female was then inspected for attached spermatophores and the water was screened for lost spermatophores by means of a dissecting microscope. The sizes of the males and of the retrieved spermatophores were measured. Images were obtained with a digital video camera (uEye, Imaging Development Systems) connected to an inverted microscope (Olympus IX71), and analyzed with the shareware Image J 1.38X. We estimated the volume of each spermatophore from its length and width assuming an ellipsoid shape.

Expt 5: sperm content in relation to spermatophore size

The final experiment was designed to estimate the total number of spermatozoa inside the spermatophores of *Temora longicornis*. We retrieved spermatophores attached to females and used either females from our lab culture (N = 30) or live females collected in the central and southern part of the North Sea in August 2010 (N = 9). To be sure that the spermatophores had not started emptying their contents, only female copepods with more than 2 attached spermatophores were used. The females have 2 genital openings, and it is believed that additional spermatophores cannot be connected to the genital openings because they are full. We modified existing DAPI protocols used for insects, bacteria and blue-green algae to stain the spermatozoa before counting (Porter & Feig 1980, LaMunyon 2001). The females were killed by freezing at −18°C. After thawing, the females were rinsed in a phosphate buffer solution (PBS, pH 7.0) and individually transferred to a microscope slide. The spermatophores not attached directly to the genital pore were separated from the female by means of fine forceps, measured, and individually placed in a 30 µl drop of PBS on a new microscope slide. The spermatophore was cut open with fine forceps to release the spermatozoa and the entire contents on the slide were transferred to a 1.5 ml centrifuge tube. The microscope slide was rinsed several times in PBS to ensure that the entire contents were transferred. The sample was diluted with PBS to 187.5 µl and centrifuged for 2 min at 2500 rpm to separate the sperm from the spermatophore. The sample was then diluted to a total volume of 200 µl with the addition of the DNA label

4,6-diamidino-2-phenylindole (DAPI; final concentration 10 µg ml^{-1}). Each sample was vortexed for 10 s and stored in the dark for at least 4 min to allow sufficient DAPI labelling. The stained sample was filtered through a 0.2 µm black polycarbonate filter and the number of spermatozoa was counted under an epifluorescence microscope (Olympus; wave length, 365 nm).

RESULTS

Longevity and age-dependent mortality

The adult longevity (mean ± SD) of virgin *Temora longicornis* was very similar between the 2 genders (females: 30.6 ± 13.4 d; males: 30.6 ± 14.6 d), as were the temporal patterns of survivorship and mortality rates (Fig. 1). The maximum individual lifespan observed among the 300 individuals of the 2 genders was 68 and 70 d for females and males, respectively. The mortality rates increased significantly with age and led to a concave shape of the log survival versus age curves (Fig. 1); hence, both male and female age and ageing become evident in the curves as elevated mortality for both genders.

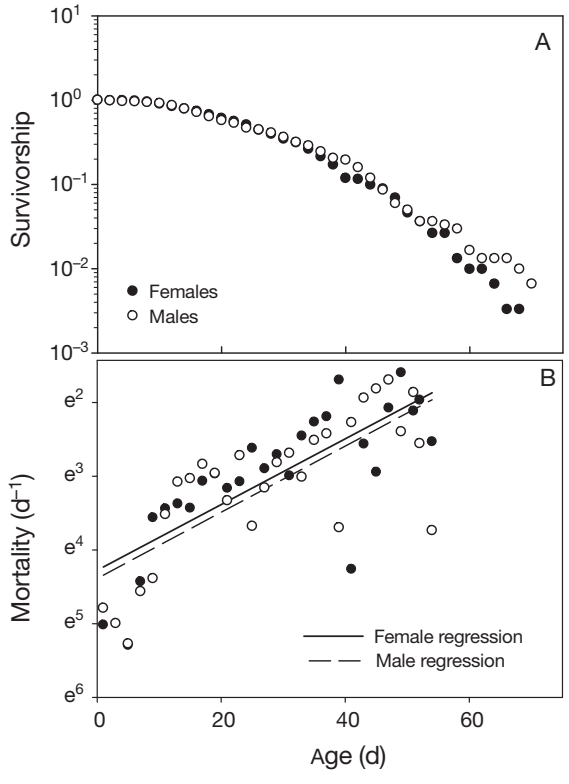

Fig. 1. *Temora longicornis*. (A) Survivorship curves for virgin females (●; N = 300) and virgin males (○; N = 300). Survivorship is defined as the fraction of individuals surviving as a function of time. (B) Instantaneous mortality rates of males and females computed for 2 d intervals as long as there were at least 10 individuals left in the cohort. Female regression: $\log_e$[mortality (d^{-1})] = −4.28 + 0.045·[age (d)] (R^2 = 0.58); male regression: $\log_e$[mortality (d^{-1})] = −4.39 + 0.045·[age (d)] (R^2 = 0.57). Both relations are statistically significant ($p < 0.001$) whereas the difference between male and female mortality patterns is not

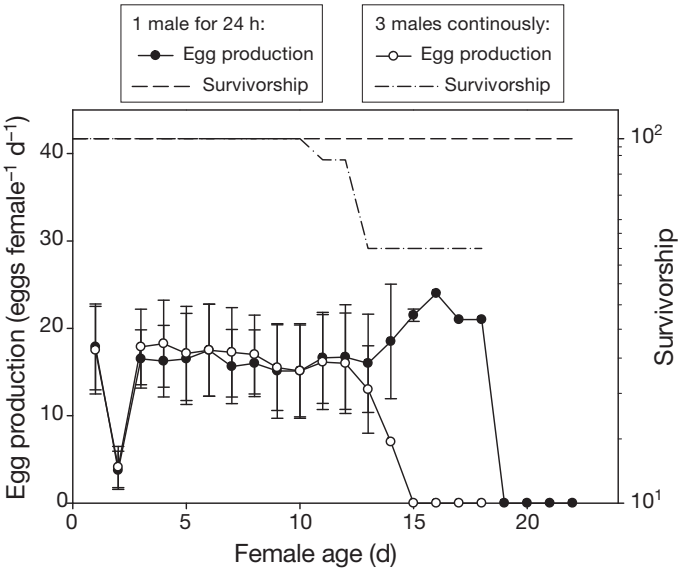

Fig. 2. *Temora longicornis*. Egg production rate (no. eggs female^{-1} d^{-1}; means ±SD) as a function of female age (d) since maturation, for females incubated with a single male for 24 h (●) and for females continuously incubated with 3 males (○) (N = 8 for each treatment). The experiment was run until the females did not produce eggs for 4 consecutive days (zero values). Only females producing eggs were included (20% of the females never produced eggs). Survivorship curves (dashed lines) during the experiment are significantly different for females with 1 male or 3 males (Mann-Whitney rank sum, p = 0.001)

Duration of the fertile period of females, and age-dependent and lifetime egg production

The fertile period of the female was short relative to the average longevity (cf. above) and varied only insignificantly between the 2 treatments, 13.9 ± 2.0 d for females incubated with a single male for 24 h and 12.3 ± 1.2 d for females incubated with males throughout the reproductive period (Fig. 2) (*t*-test, p = 0.070). The lifetime egg production was also similar between the 2 treatments (218 ± 76 vs. 191 ± 41 eggs, p = 0.388), which suggested that cessation of egg production was not due to a shortage of sperm. In both

treatments, 20% of the females did not produce any eggs during the experiment and therefore they are not included in the results. Repeated mating led to a higher mortality in females (Fig. 2).

Age-dependent male mating performance and fertility

Males mated only during the first 8 d after they matured, and the decline in mating performance had already started after 5 d (Fig. 3A). Again, this fertile period is short relative to the average longevity of males (cf. above). Also, the female's offspring production is related to the age of the male with which she had mated: young fathers sire significantly more offspring per mating than do older ones (Fig. 3B). The size of the female will also affect her offspring production (cf. below), which thus accounts for the large variance in offspring production, but all females in this experiment were virgins and of the same age (matured within 24 h). Therefore, the general decrease in offspring production is a result of the age of the male.

Size-dependent male mating performance and fertility

Large males were superior to small males in terms of reproductive performance in 2 ways: large males both mated more frequently (Fig. 4A) and sired more offspring per successful mating than small males (Fig. 4B).

Size-dependent female reproduction

To examine whether the size of the female has an effect on total lifetime offspring production and egg production rate, data for all females were compiled. Large females had a higher total lifetime offspring production compared with small females and they also had a higher instantaneous egg production rate (Fig. 5). The size effect was substantial: a 30% increase in female body length led to a 2- to 3-fold increase in egg production, which suggests that the egg production is approximately proportional to female body volume.

Spermatophore size and sperm content

Male size and spermatophore size varied substantially, and large males produced larger spermatophores than did small males (Fig. 6A). In fact, a modest increase in male prosome length (750 to 780 µm) led to a 4-fold increase in spermatophore volume. Larger spermatophores contained more sperm cells, although sperm content was far from proportional to spermatophore volume (a 4-fold increase in volume led to an increase in sperm count from 1000 to 1300) (Fig. 6B). This modest increase in spermatozoa number with increased volume suggested that large spermatophores either have a disproportionally larger swelling capacity to create hydrostatic pressure for sperm transferral or contain disproportional amounts of additional substances that are transferred with the sperm. The average number of spermatozoa inside a

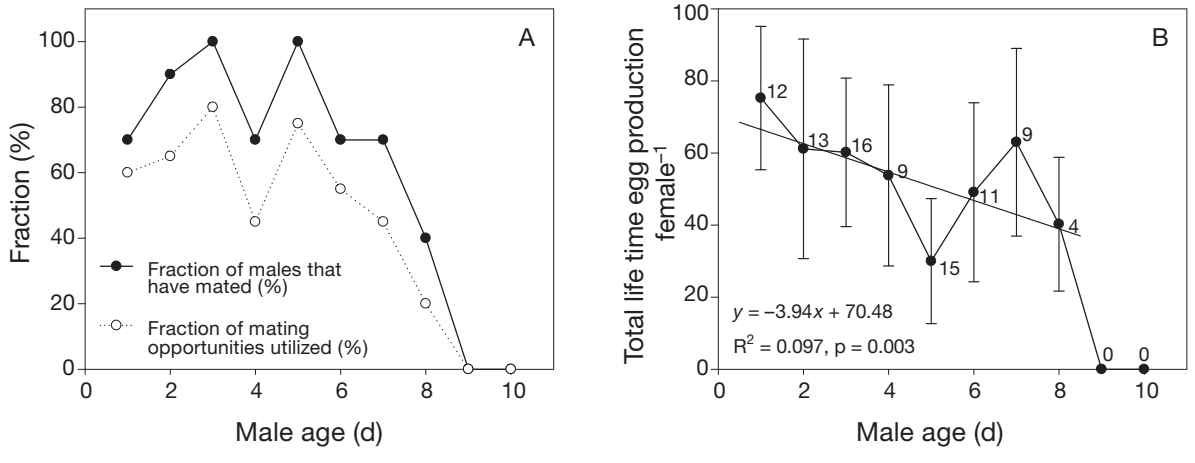

Fig. 3. *Temora longicornis*. Male mating performance and fertility. (A) The fraction of males (out of 10 males) that had mated as a function of male age since maturation (●) and the fraction of mating opportunities used (fraction of 20 females fertilized) as a function of male age (○). (B) Total life time offspring production per fertilized female ± SD as a function of male age since maturation (d). The numbers correspond to the number of fertilized females out of 20. The trend line is a linear regression that used individual values (not averages) for Days 1 to 8; the slope of this regression is statistically significant (p = 0.003) and demonstrates that young males sire more offspring per mating than do old males in the reproductive period

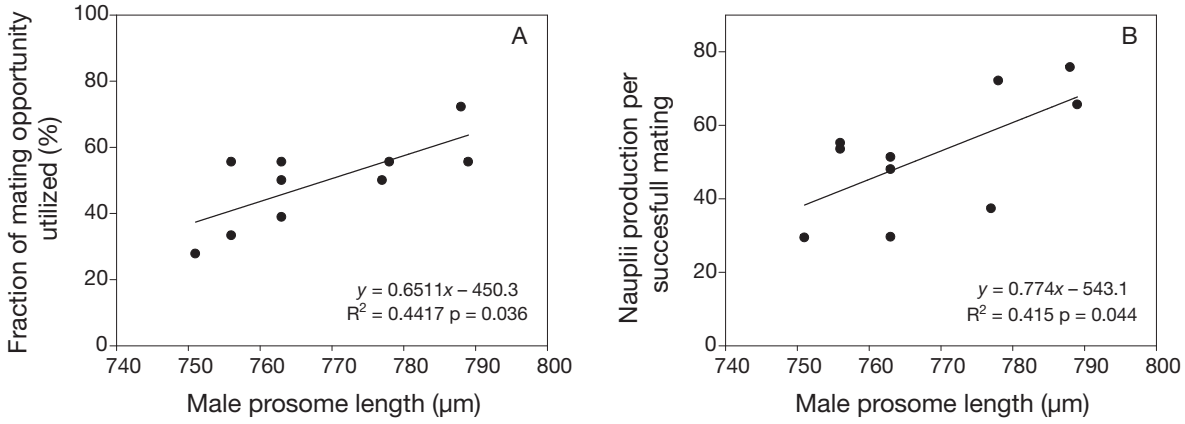

Fig. 4. *Temora longicornis*. (A) Fraction of mating opportunities that males had used (%) as a function of male size. (B) Nauplii production per successful mating as a function of the father's size. The experiment lasted 8 d and each male (N = 10) was offered 2 new virgin females every day. Same experimental individuals as in Fig. 3

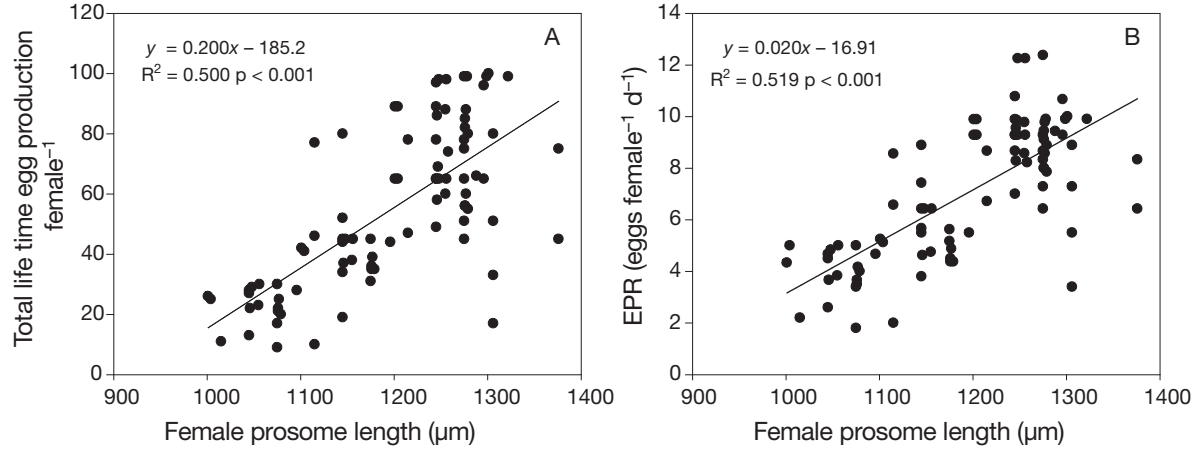

Fig. 5. *Temora longicornis*. (A) Lifetime egg production as a function of the female prosome size (N = 89). (B) Egg production rate (EPR) as a function of female prosome length. The egg production was followed for 10 d

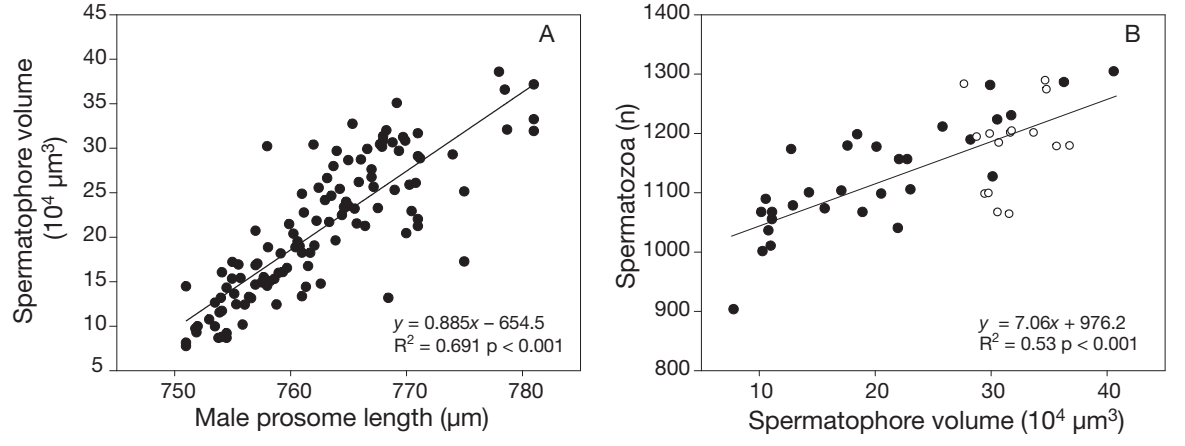

Fig. 6. *Temora longicornis*. (A) Spermatophore volume (10^4 µm^3) as a function of male size (µm) (N = 115). (B) Number of spermatozoa as a function of the spermatophore volume (10^4 µm^3) in a laboratory culture (●) (N = 30) and in field samples (○) (N = 15)

single spermatophore was 1126 ± 92, which corresponds to about 5 times the number of eggs that a female can produce after a mating event.

DISCUSSION

Our results showed 3 clear findings. (1) Ageing effects were evident. Mortality rate increased with age, and fertility decreased rapidly with age. This latter effect was stronger in males than females. Also, in both genders, the reproductive period was significantly shorter than the average longevity. (2) Several aspects of reproductive performance increased with size in both males and females: large females produced more offspring than small ones, and large males mated more often, produced larger spermatophores containing more sperm cells and sired more offspring. (3) Repeated mating was not only potentially advantageous (e.g. in terms of higher genetic variability) for females, but could also have had disadvantages as it led to higher mortality. While the absolute rates and durations reported in this study applied to our laboratory culture and conditions of the experiments, we believe that the patterns apply generally to this species.

Young males of *Temora longicornis* have a higher mating success and a higher reproductive success compared with old males. Whether this is due to females preferring young males, young males performing better (better sperm quality and quantity) or young males being more efficient in capturing and mating with females is unknown. In theory, female preference for males of particular ages is thought to be maintained largely through the benefits accrued by choosy females (Manning 1985, Hansen & Price 1995, Kokko 1998, Beck & Powell 2000, Proulx et al. 2002). One possible explanation for the elevated offspring production in young males is declining sperm number in old males, or increasing damage to the DNA or the spermatozoan cell membrane as seen in other species (Vishwanath & Shannon 1997, Irvine et al. 2000). Theory predicts that sperm quality declines with age owing to the accumulation of de novo mutations in the germline cells (Hansen & Price 1995, 1999). Females mating with old males may therefore suffer reduced fertility. Sperm age may be independent of male age; successful males may replenish their sperm frequently and so have better fertilization success than less successful competitors regardless of age (Siva-Jothy 2000). The old males in this experiment were not virgins and were offered new females daily. Therefore, the possibility is low that their sperm is old, and the observed decline in male success with age is probably an effect of male age. Direct trade-offs between fitness components and age-specific differences in survival may reduce the fertility of older males and instead promote the evolution of female preference for young males and those of intermediate age (Hansen & Price 1995, 1999, Kokko 1997, Beck & Powell 2000, Beck et al. 2002). Males in particular, but also females, can survive beyond their reproductive time period (30.6 versus 8 or 15 d). Such post-reproductive life may be an evolutionary adaptation to low mate-encounter rates if the reproductive performance is maintained in the absence of matings, as shown in another pelagic copepod species (Ceballos & Kiørboe 2011).

Size is a heritable trait in copepods (McLaren 1976, McLaren & Corkett 1978) and females that choose large males may therefore sire larger sons and daughters that likewise sire more offspring (Weatherhead & Robertson 1979). Morphologically, body size is positively correlated with reproductive organ size and total number of gametes, and it is well documented in comparisons between species that egg production rates in copepods, like in many other organisms, increase with size (Kiørboe & Hirst 2008). Comparisons within species are rare and the relationship observed here for *Temora longicornis* is much more pronounced than comparisons between species, where numerical egg production increases with body mass (or volume) to a power of 0.2 (Kiørboe & Hirst 2008). The size dependency of reproductive performance in male copepods has previously been examined in only one other species, *Acartia tonsa* (Ceballos & Kiørboe 2010), and is in accordance with our observation in *T. longicornis* that large males produce larger spermatophores and sire more offspring per mating than do small males. We showed that this is related to a higher content of sperm cells in the spermatophores.

Multiple mating (polyandry) in female insects is reported to increase fecundity and egg viability across a range of taxa (Arnqvist & Nilsson 2000). In copepods, polyandry is often observed both in laboratory and field populations (many attached spermatophores), and it is typically assumed that copepods belonging to the superfamily Centropagoidea, such as *Temora longicornis*, need to mate several times during their life because they lack dedicated sperm storage organs (spermathecae). This is, for example, the case in a sibling species, *T. stylifera* (Barthélémy et al. 1998). However, a female of *T. longicornis* receives sufficient sperm in 1 mating to fertilize all the eggs she produces in her life. In our

study, the lifetime egg production was 203 ± 62 eggs and the number of sperm per spermatophore ranged from 900 to 1300. This means that the female only use 15 to 22% of the sperm in 1 spermatophore to fertilize all of her eggs. Consistent with this, we find that multiple matings in *T. longicornis* do not increase the reproductive output in the females. We did not examine whether the offspring from one or several mating opportunities had different fitness levels, e.g. in terms of higher survival, and whether multiple mating secures higher genetic variation or allows for cryptic mate choice leading to fitter offspring. It is unclear whether cryptic mate choice is at all feasible in copepods because the genital opening may be blocked by the spermatophore or in some species by a cement plug, but there is molecular evidence in one such species, *Lepeophtheirus salmonis*, that a batch of eggs may in fact be sired by several fathers (Todd et al. 2005). However, there is a mortality penalty related to matings; repeated mating leads to a higher mortality in females (Fig. 2), probably owing to elevated energy consumption or damage or injury by the male when mating. This has also been demonstrated for another copepod species (*Oithona davisae*; Ceballos & Kiørboe 2011) and in several insect species (Campbell 2005, Wenninger & Hall 2008); thus, any advantages of multiple matings have to be traded off against the mortality penalty. In some species of insects, the seminal fluid contains substances that increase the male's relative paternity at the expense of female fitness (Chapman et al. 1995, Simmons 2001, Gillott 2003), which explains the elevated mortality of females that mate multiple times (Arnqvist & Nilsson 2000). The volume of the spermatophores and the volume of the total number of spermatozoa leave plenty of space in the spermatophores for seminal fluids.

Titelman et al. (2007) reviewed indirect evidence to suggest that mating in pelagic copepods was nonrandom and the result of mate choice. The strong age- and size-dependency of the reproductive output in both males and females and the mortality penalty to mating, in at least the females, are conducive to the development of sexual selection through mate choosiness. Necessary conditions for the development of choosiness are a high mate encounter rate, a cost to mating (in terms of elevated mortality or lost future mating opportunities) and, finally, that mates are of different quality and being choosy implies a fitness benefit (Kokko & Monaghan 2001, Shuster 2007). We have shown for *Temora longicornis* that mating with large, young partners implies higher offspring production in both males and females, and have provided evidence (higher mating success of young, large males) that is consistent with mate choosiness. Our study, together with recent observations of sexual selection through mate choosiness in other species of copepods (Ceballos & Kiørboe 2010), adds further evidence that sexual selection is an important determinant of the behavioral ecology and population dynamics of pelagic copepods.

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CHAPTER 4

Identification and characterization of six microsatellite primers for the calanoid copepod *Temora longicornis*

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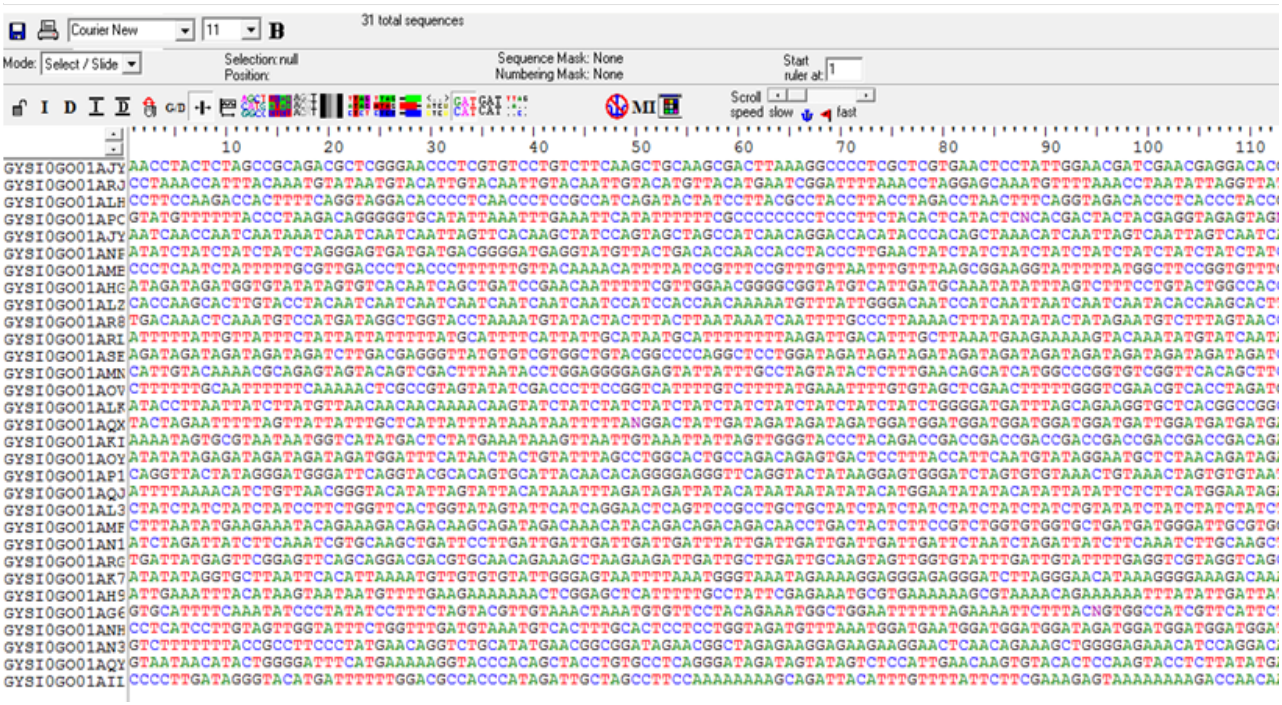

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Abstract

Next generation sequencing (NGS) allows rapid development of genetic markers for investigating the ecology and evolution of a species. In the present study we used NGS for isolation of microsatellites for the calanoid copepod *Temora longicornis*, a commonly found species in the northern hemisphere. 46 *in silico* primer pairs were designed, 17 primer pairs were tested with six loci providing consistent amplification, were polymorphic and could be reliably genotyped. The average number of alleles was 6.5 and average observed and expected heterozygosity of 0.55 and 0.64, respectively. No linkage disequilibrium between pairs of loci was observed. These markers are expected to be a useful tool in parentage and mating systems analysis in *Temora longicornis.*

Keywords: copepods, microsatellites, next generation sequencing, *Temora longicornis*

Introduction

Copepods are among the most abundant metazoans on the planet (Humes 1994, Turner 2004) and play a ubiquitous role in the marine food web. Despite this, microsatellite loci have only been developed for relatively few species (Harrison et al. 2004, Zeller & Reusch 2004, Todd et al. 2004, Provan et al. 2007, Ferrada et al. 2011). The calanoid copepod *Temora longicornis* is a commonly found species in many coastal areas of the Northern hemisphere (Fransz et al. 1992, Peterson & Kimmerer 1994, Williams et al. 1994). Recent findings (Ceballos & Kiørboe 2010, Sichlau & Kiørboe 2011) show that copepods in the lab can be very choosy with respect to selection of mating partner, thus strongly suggesting an adaptive value and ecological importance of mate choice in marine zooplankton. Sexual selection has proven to be a very strong determinant of the evolution of the morphology, behaviour of individuals, population biology, speciation and biodiversity in many others species (Emlen & Orin 1977, Andersson 1994, Schluter 2001).

Molecular genetic analyses of parentage could provide useful insights into the many open questions regarding mating systems in zooplankton, which is important in understanding the functional ecology and population dynamics of this group of organisms. Here we report the identification, primer development and characterization of microsatellite loci for *Temora longicornis.*

Method

Temora longicornis was originally sampled in the Kattegat area (Baltic Sea) and kept in continuous cultures at 14 °C and darkness in the laboratory at the National Institute of Aquatic Resources in Charlottenlund (Denmark). Before DNA extraction animals were incubated for 4 hours in 0.22 µm filtered seawater to empty their guts and prevent DNA contamination from ingested food. Each individual were cleaned in HPCL water before DNA extraction. 454 next generation sequencing was used to identify microsatellite markers for *Temora longicornis*. As a source for marker development, 5.44 µg of high molecular, RNA-free DNA (concentration 171.8 ng/µl) was extracted from 300 pooled individuals of *T. longicornis* with the E.Z.N.A® Tissue DNA Kit (Omega Bio-Tek), using the protocol for isolation of total DNA from animal tissue. Construction of standard genomic library on 454 FLX genome sequence (Roche, Basel, CH) was done using Titanium chemistry. Sequencing was performed by GATC Biotech AG (Konstanz, Germany). A total of 12,206,429 bases and 30,851 sequences were obtained with an average sequence length of 395 bases. Tandem repeats with flanking regions were identified *in silico* by using the Tandem Repeats Finder (TRF) software, version 4.04 (http://tandem.bu.edu/trf/trf.html) (Benson 1999), with the following options: alignment parameters, "2,7,7", minimum alignment score to report repeat, 70, maximum period size, 4 base-pairs. From 761 individual sequence reads, PCR primers for 46 microsatellites loci were designed *in silico*

with the Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) (Koressaar & Remm 2007, Untergasser et al. 2012).

As copepods and particular early life stages (eggs and nauplii) contains very little tissue, four different DNA extraction methods (E.Z.N.A® Tissue DNA Kits (Omega Bio-Tek), NucleoSpin-Tissue-Kit (Macherey-Nagel), Wizard Genomic DNA Purification System kit (Promega) and Chelex-100 resin (BioRad)) were tested in order to assess the best PCR amplification. All four extraction methods were performed according to the manufacturer`s instructions for isolation of total DNA from animal tissue. Comparison among the four different DNA extraction methods revealed the greatest success in PCR amplification for samples extracted by Chelex-100 resin. Similar results have been reported previously for other tissues (Dolnik et al. 2009, Mendoza et al. 2012).

Primers were optimized using a sample of 28 adult specimens of *T. longicornis*. Total genomic DNA was extracted from *T. longicornis* with Chelex-100 resin, using the protocol for isolation of total DNA (Estoup et al. 1996). Amplification reactions were carried out in a multi-block PCR system (MBS) thermocycler (ThermoHybaid Ashford, UK). PCR amplifications were performed using one fluorescent labeled primer (forward) and one unlabeled (reverse). The PCR amplification contained a final volume of 6 µl, which included 50 to 100 ng of genomic DNA, 0.2 µM of each primer, 2,25µl RNase-free water and 3,125 µL Multiplex PCR Kit (QIAGEN GmbH) (containing hot-start DNA polymerase, PCR buffer and dNTP mix).The following temperature profile was used: 15 min initial heating at 95°C followed by 39 cycles of 30 s denaturation at 94°C, 3 min annealing at 57-62°C and 60 s extension at 72°C. A final extension step was run at 60°C for 30 min. The fragments were analyzed on an ABI 3130 Genetic Analyzer (Applied Biosystems, USA). Microsatellites loci were scored using the software GeneMapper (GeneMapper Software v4.0; Applied Biosystems). The software PowerMarker version 3.25 (Liu & Muse 2005) was used to calculate basic summery statistics for each locus [size range, number of individuals scored (N_1), number of alleles per locus (N_a), deviations from Hardy-Weinberg Equilibrium proportions (P_{HW}), observed (H_o), and expected (He) heterozygosities]. The presence of null alleles for each locus was checked using Micro-Checker version 2.2.3 (Van Oosterhout et al. 2004). Test for linkage disequilibrium were performed using GENEPOP 4.2.1 (Raymond & Rousset 1995, Rousset 2008).

Table 1. Primer sequences and characteristics of six *Temora longicornis* microsatellite loci. N_1, number of individuals scored; N_a, numbers of alleles; T_a, optimum annealing temperature; H_O, observed heterozygosity; H_E, expected heterozygosity; P_{HW}, P value of the test for HWE

Locus	N_1	Primer Sequence (5'-3')	Repeat Motif	N_a	Alleles size range (bp)	T_a	H_o	H_E	P_{HW}
Tl01	14	F:AAATGCCGGAGTCAAAAATG R:ACCTCTGGCTATCCCTCGAT	$GATA_{(9)}$	5	114-146	55	0.6429	0.7194	0.134
Tl06	22	F:CCATTTTCGTCAACCCGTAA R:ACCTGCTGCGTCAACGAC	$TATC_{(11)}$	11	129-249	62	0.9545	0.7593	0.167
Tl07	22	F:TGTCGGTTCACAGCTTCTTG R:CAAGCAAAGACGGTTTTGAG	$ATCA_{(8)}$	2	106-118	57	0.1818	0.2355	0.351
Tl08	23	F:ATCTTATGTTAACAACAACAAAACAAG R:AAGTGTTAAAGAGAGGCTTGGA	$TATC_{(10)}$	4	139-155	55	0.3913	0.5321	0.255
Tl11	28	F:TGGATACAGGAGAGGGCAAA R:TGGAAGTGTGGAGCCACTAA	$ATAG_{(10)}$	4	137-153	57	0.7857	0.6818	0.699
Tl14	14	F:GCCAGACAGAGTGACTCCTTTA R:TTTTGGGTTATTCAACCACTCT	$AGAT_{(18)}$	13	173-209	62	0.3571	0.8827	0

Results and discussion

Of the tested 17 microsatellite primer pairs 11 were discarded either due to low levels of polymorphism, insufficient information content or difficulty to amplify or score, leaving a total of six informative and consistently amplified loci (table 1). Of these 6, the total number of observed alleles per locus ranged from 2 (Tl7) to 13 (Tl14), with an average of 6.5 alleles per locus.

Observed heterozygosity (H_o) ranged from 0.18-0.95, while expected heterozygosity ranged from 0.24-0.88 (table 1). Results from PowerMarker revealed that one locus (Tl14) deviated from Hardy-Weinberg equilibrium (HWE). The results from Micro-Checker showed signs of null alleles for locus Tl14, while there was no evidence for scoring error due to stuttering or mating systems. Accordingly, this locus should be applied with caution in population genetic studies and parentage analysis (Dakin & Avise 2004). No significant genotypic disequilibrium between loci was detected.

The microsatellites markers described in this study provide a valuable tool for wide application in ecological and population genetics assessments of *Temora longicornis*. In particular, molecular genetic parentage analyses could provide completely novel insights into mating systems of the species. Inferring parentage in

natural populations of zooplankton is important for understanding the (mating) behaviour, functional ecology, population dynamics and evolution of this vastly distributed and ecologically essential organismal group.

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CHAPTER 5

Mating success and sexual selection in a pelagic copepod, *Temora longicornis*: Evidence from paternity analyses

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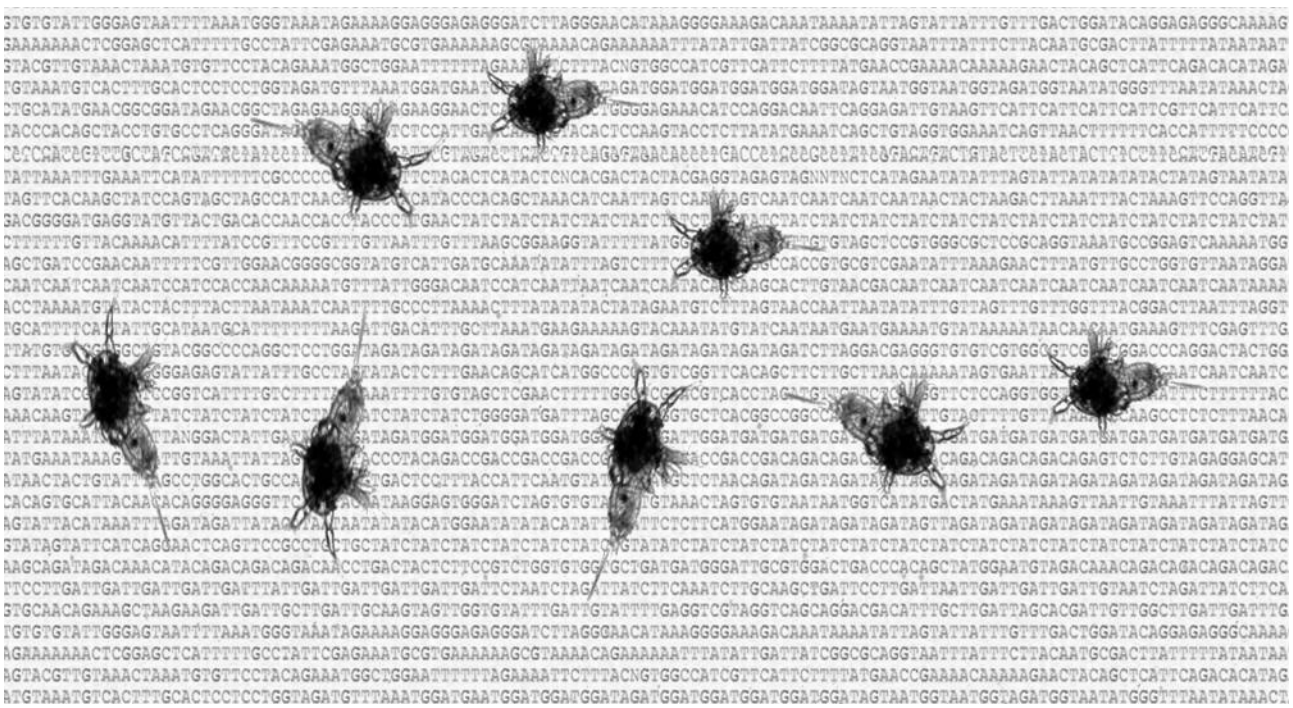

Photo: Rodrigo Gonçalves & Mie Hylstofte Sichlau

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Abstract

Knowledge about mating patterns is essential for understanding and explaining rates of reproduction and genetic potential of copepods populations**.** The aim of this study was to examine *i*) the occurrence of multiple paternity in *Temora longicornis*, *ii*) the effect of multiple paternity (if present) on the females reproductive output, and *iii*) whether mating is random or some individuals have a higher than average chance of fertilizing or being fertilized ('super individuals'). We show that multiple paternity is common in this copepod species, that females benefit from multiple matings by increased offspring production, and that a relatively small fraction of the males and females in a population account for most of the offspring production. In both males and females, mating is non-random. Superior individuals with a higher than average mating success were identified both among females and among males.

Keywords: Sexual selection, mate choice, mating, multiple paternity, microsatellite, reproductive success, copepods

Introduction

Any trait involved in enhancing mating and ultimately fertilization success can be favoured by sexual selection. Sexual selection is an important process for micro- and macro evolution (Schluter 2001) and has proven to be a very strong determinant of the evolution of the morphology, behaviour, speciation and overall biodiversity (Emlen & Orin 1977, Andersson 1994, Schluter 2001, Kokko & Rankin 2006). In addition sexual selection can also affect population dynamics (Kokko & Rankin 2006).

Copepods are among the most abundant metazoans on the planet (Humes 1994, Turner 2004) and they play an important role in the marine food web. Many aspects of their ecology have consequently been studied, including details of their reproductive biology and mating behaviour (Uchima & Murano 1988, Doall et al. 1998, Yen et al. 1998, Weissburg et al. 1998, Buskey 1998, Bagøien & Kiørboe 2005, Titelman et al. 2007, Dur et al. 2011, Seuront 2013, Heuschele et al. 2013). Yet very little is known about the significance of sexual selection for the evolution and ecology of this key group of animals (Titelman et al. 2007). The lack of studies may be due to the difficulty of perceiving the cues that may be used by these small pelagic organisms in choosing mates, and to the implicit assumption that mating is encounter-limited in pelagic copepods (Kokko & Mappes 2005, Titelman et al. 2007). However, recent studies have shown that mate encounter rates in pelagic copepods are typically high (Kiørboe & Bagøien 2005), and that sexual selection may operate via mate choosiness, with both males and females preferring to mate with large and young partners (Ceballos & Kiørboe 2010, Sichlau & Kiørboe 2011). However, cryptic mate choice and post-copulatory sperm selection may be equally or more important components of sexual selection (Olsson et al. 1996, Tregenza & Wedell 2002, Bretman et al. 2004, Simmons 2005), but cannot be easily be observed and quantified by experimentalists and their significance consequently unknown for pelagic copepods.

Polyandry (i.e., females mate with more than one male) and polygyny (i.e., males mate with more than one female) occurs across a wide range of environments and taxa, including insects (Arnqvist & Nilsson 2000), birds (Birkhead & Møller 1995), mammals (Gomendio et al. 1998), mollusks (Dupont et al. 2006), fish (Feldheim et al. 2002, Dibattista et al. 2008, Barbosa et al. 2010), and reptiles (Laloi et al. 2004, Uller & Olsson 2008). The development of new molecular techniques has revolutionized our understanding of mating systems in nature, and species thought to be monogamous have revealed moderate to high levels of polyandry (Birkhead & Møller 1998, Simmons 2005, Parker & Birkhead 2013). Male benefits of multiple mating are well known (Bateman 1948, Arnqvist & Nilsson 2000), while female benefits of polyandry and multiple paternities remain an open question (Andersson 1994, DiBattista et al. 2008). In many species, females mate with more than one male to receive direct material benefits (e.g., fertilization assurance and material benefits (also called nuptial gifts) provided in spermatophores) and indirect genetic benefits (e.g.,

higher offspring diversity and higher offspring viability) and to avoid genetic incompatibility and inbreeding (Arnqvist & Nilsson 2000, Birkhead 2000, Jennions & Petrie 2000, Arnqvist & Rowe 2005, Simmons 2005). Polyandry in female insects is reported to increase fecundity and egg viability across a range of taxa (Arnqvist & Nilsson 2000). Genetic analyses of mating systems in marine organism are relatively scarce, despite the extensive potential for female promiscuity and polyandry in the aquatic environment (Kamel et al. 2010). In pelagic copepods, polyandry is often observed both in laboratory and field populations, as evidenced by females carrying multiple spermatophores (Katona 1975, Jacoby & Youngbluth 1983). Females of the copepod *Temora longicornis* are often seen carrying several attached spermatophores simultaneously (Mie Hylstofte Sichlau pers. obs. of up to 70 attached spermatophores in the lab). Each spermatophore represents a mating event and hence the presence of multiple spermatophores implies frequent re-mating, even after they may have been fertilized. However, it is unclear whether in copepods the first spermatophore blocks the genital opening, thus preventing the sperm from subsequent spermatophores from fertilizing the eggs (Miller 1988). There is molecular evidence in one ectoparasitic copepod, *Lepeophtheirus salmonis*, that a batch of eggs may be sired by several fathers (Todd et al. 2005). However multiple paternity has otherwise never been investigated in copepods, and it is unclear what advantage, if any, females may have from multiple matings. Sichlau and Kiørboe (2011) found that repeated matings in *T. longicornis* did not increase the reproductive output in the females. In this species, one insemination is sufficient to fertilize most or all of the batches of eggs that the female subsequently produces (Sichlau & Kiørboe 2011).

In mating systems with mixed paternity broods, male age and the body size of both sexes frequently play an important role in determining reproductive success (Clutton-Brock 1988, Levitan 1991). Age and size of both female and male copepods have been shown to be important factors affecting copepods mating behaviour (Ceballos & Kiørboe 2010, Sichlau & Kiørboe 2011). Many marine organisms are difficult to observe while mating, and it is usually impossible to determine which individuals are successful (Karl 2008). This applies to copepods as well, and previous experiments to examine mating in copepods have been designed as one-male-to-one female incubations or as incubations where the successful males cannot be identified. Genetic tools, such as DNA based microsatellites allow paternity assignment of animals and recently microsatellite markers have been developed for a few species of copepods (Sichlau et al. submitted , Harrison et al. 2004, Zeller & Reusch 2004, Todd et al. 2004, Provan et al. 2007, Ferrada et al. 2011) opening new experimental possibilities.

The aim of this study was to examine *i*) the occurrence of multiple paternity in *Temora longicornis, ii*) the effect of multiple paternity (if present) on the females reproductive output, and *iii*) whether mating is random or some individuals have a higher than average chance of fertilizing or being fertilized (‘super individuals’). We show that multiple paternity is common in this copepod species, that females benefit from multiple

matings by increased offspring production, and that a relatively small fraction of the males and females in a population account for most of the offspring production. We discuss these findings in the light of sexual selection.

Material and Methods

The calanoid copepod *Temora longicornis* is a mm-sized copepod common in coastal areas of the Northern hemisphere (Fransz et al. 1992, Peterson & Kimmerer 1994, Williams et al. 1994). At typical population densities mate encounter rates are high and facilitated by pheromone signalling, thus mating is rarely encounter limited (Doall et al. 1998, Kiørboe & Bagøien 2005). For our experiments we used animals recently collected in the Kattegat area (Baltic Sea) and kept in continuous cultures at 14°C and darkness on a mixed diet of phytoplankton. All animals used in the experiments were raised from eggs produced in the culture.

All experiments were conducted in darkness, at 14°C and a salinity of 32. We only used virgin adults in the experiments. Prior to an experiment a sufficient number of animals were isolated from the culture as late copepodites (Stages CIV to CV) and incubated individually in 69 mL bottles till maturation. Copepods for experiments (*see* below) were incubated in 630 mL screw-cap bottles that were sealed without a head space and rotated on a plankton wheel (1 rpm) to avoid the sedimentation of food particles. Rotation does not affect mating success in copepods (Choi & Kimmerer 2009). We used four independent populations to avoid inbreeding and to secure the highest possible genetic variation among individuals. During all incubations food was supplied in excess (~ 500 μg C L^{-1}, phytoplankton of species *Rhodomonas salina, Thalassiosira weissflogii* and *Prorocentrum minimum* (Dam & Peterson 1991)).

Experimental setup

We conducted two experiments to examine individual variability in reproductive success. In experiment 1, we examined the effect of male size on their reproductive success and in experiment 2 we examined the effect female size and male age on their respective mating success. Mating success was quantified as the number of females fertilized (males), the number of offspring produced (both sexes), and the number of fathers that sired the offspring of a female.

Incubations

Experiment 1:

10 virgin females and three virgin males were placed in each experimental bottle (n = 7). All females were of the same size (length) (coefficient of variation: mean 0.0327, range 0.025-0.043) and age (moulted to adult within 24 h). The males in each incubation bottle were of different size (coefficient of variation: mean 0.0917, range 0.051-0.169) and of same age (moulted to adult within 24 h). In one of the experiments the males were noticeably smaller (0.4-0.6 mm), but were all able to mate successfully and they are therefore included in the dataset.

Experiment 2:

13 virgin females and four virgin males were placed in each experimental bottle (n = 3). All females in each incubation bottle varied in size (coefficient of variation: mean 0.0724, range 0.070-0.075) but were of the same age (moulted to stage CVI within 24 h). The males were of the same size (coefficient of variation: mean 0.0227, range 0.013-0.028) but of different age: 2 young males (matured within 24 h) and 2 old males (matured > 6 days earlier). Young and old males were identified from image identification (before and after pictures) from images obtained with a digital camera (EOS 50D, Canon) connected to a microscope (Olympus SZX9).

Production of fertilized eggs

After 30 h males were collected for DNA extraction while the females were transferred individually to 630 mL incubations bottles with food (Exp. 1: n=68, Exp. 2: n=32). Every subsequent day the females were transferred to new bottles and eggs produced during the preceding 24 h were collected on a 50 μm filter, counted, and placed in 10 mL incubations wells. The eggs were incubated until the nauplii had hatched and had reached nauplius stage 2, at which time they were collected for DNA extraction. Females were collected after 5 to 10 days. The size of the males and females were measured from images obtained with a digital camera (EOS 50D, Canon) connected to a microscope (Olympus SZX9). Before DNA extraction animals were incubated for 4 hours in 0.22 μm filtered seawater to empty their guts and prevent DNA contamination from ingested food. All animals were frozen at -18°C and DNA was extracted within 24 hours.

Molecular analysis

As copepods and particularly early life stages (eggs and nauplii) contain only small quantities of DNA some of the gene amplifications failed or were too faint despite several genotyping attempts. These samples were removed from the analyses. Each individual was cleaned in HPCL-water before DNA extraction. Total

genomic DNA was extracted with Chelex-100 resin, using the protocol for isolation of total DNA (Estoup et al. 1996) as M.H. Sichlau unpubl. found the greatest success in PCR amplification for samples of this copepod species extracted using this approach. We assigned paternity to offspring using 5 variable microsatellites DNA loci (Tl01, Tl06, Tl07, Tl08 and Tl11, Sichlau et al submitted). Amplification reactions were carried out in a multiblock PCR system (MBS) thermocycler (ThermoHybaid Ashford, UK). PCR amplifications were performed using one fluorescent labeled primer (forward) and one unlabeled (reverse). The PCR amplification contained a final volume of 6 µL, which included 50 to 100 ng of genomic DNA, 0.2 mol L^{-1} of each primer, 2,25 μL RNase-free water and 3.125 μL Multiplex PCR Kit (QIAGEN GmbH) (containing hot-start DNA polymerase, PCR buffer and dNTP mix).The following temperature profile was used: 15 min initial heating at 95°C followed by 39 cycles of 30 s denaturation at 94°C, 3 min annealing at 57-62°C and 60 s extension at 72°C. A final extension step was run at 60°C for 30 min. The fragments were analyzed on an ABI 3130 Genetic Analyzer (Applied Biosystems). Microsatellites loci were scored using the software GeneMapper (GeneMapper Software version 4.0; Applied Biosystems). The software PowerMarker version 3.25 (Liu & Muse 2005) was used to calculate basic summery statistics for each locus [number of alleles per locus (N_a), deviations from Hardy-Weinberg Equilibrium proportions (P_{HW}), observed (H_o), and expected (He) heterozygosities]. The presence of null alleles for each locus was checked using Micro-Checker version 2.2.3 (Van Oosterhout et al. 2004). Test for linkage disequilibrium were performed using GENEPOP 4.2.1 (Raymond & Rousset 1995, Rousset 2008).

Paternity analysis

Parentage analysis was conducted in COLONY 2.0.4.7 (Jones & Wang 2010) which implements a maximum-likelihood method to assign parentage. Given the genotypes of the nauplii, of their known mothers and of the potential fathers, the paternity was assigned to the male with the highest log-likelihood ratio (LOD). In the analyses, we assumed that both females and males were polygamous, since COLONY allows either monogamy or polygamy, not both. In order to be conservative we accepted only those paternities assigned with higher than 95 percent confidence in COLONY. This approach consolidate that we don't overestimate the degree of multiple paternity in the population or attribute false fitness to either males or females.

Statistical analyses

When comparing the reproductive output from females that had a single sire and females that had multiple sires to their offspring, we used a generalized linear model (GLM) for Poisson distributed data.

Experiment 1: Here we examined whether mating was random, and whether the size of the male has an effect on his chances to mate. The data were analyzed statistically using a GLM with mixed effects. For each male *i* and female *j* that had the opportunity to mate, we considered the response if they did in fact mate (R_{ij}=1) or not (R_{ij}=0). The probability of male *i* mating female *j*, p_{ij}, is modelled as

$$\text{logit } p_{ij} = a + b*L_i + e_j$$

where the logit function is logit p = log(p) - log(1-p), L_i is the length of male i, and e_j is a random effect of female j, assumed Gaussian with mean 0. We assume independence among females e_j, as well as (conditional) independence among mating responses R_{ij}. Parameter estimates are a=-12.1 and b=13.1 with standard errors 2.9 and 3.8, respectively, while the standard deviation on e[j] is estimated as sigma=3.0. The parameters were estimated using function lmer in package lme4 (version 0.999999-2) in R (version 3.0.1). Significance tests were performed using likelihood ratio tests, using function ANOVA in R package stats (version 3.0.1).

Experiment 2: Here again we examine whether mating was random, and we tested for effects on mating chance of the age of the male and the size of the female. We performed a logistic regression, where the response variable indicates whether mating between a given male and a given female takes place. As covariates we considered female length, male length, and male age, categorized as "young" or "old". We included additional random effects, one for each female individual and one for each male individual. The resulting model is thus a generalized linear mixed model, of the binomial family. The link function used is the logit function. Parameters were estimated using function glmer from package lme4 (version0.999999-2) in R (version 3.0.1). To identify significant effects, model selection was performed based on the corrected Akaike Information Criterion, using R function dredge from package MuMIn. We verified our model selection by testing the best candidates using chi-square tests, and the significance of the random effects was assessed using likelihood ratio tests.

Results

Microsatellites Markers and parentage analysis

The number of observed alleles per locus ranged from 2 (Tl7) to 14 (Tl6), with an average of 6.4 alleles per locus. Observed heterozygosity (H_o) ranged from 0.205-0.825, while expected heterozygosity ranged from 0.219-0.797 (Table 1). No evidence of genotypic disequilibrium between loci was detected and all loci were in Hardy-Weinberg equilibrium. Micro-Checker did not find any evidence for the presence of null alleles for any locus. Accordingly, the loci fulfil the quality criteria, i.e., high levels genetic variability, independence of loci and no non-amplifying alleles or PCR artefact, for parentage assignment. Parentage analysis was successfully carried out for 871 nauplii from 55 females and 34 males. However seven of the males and six of the females mating success might be underestimated due to low assigning power in COLONY. However in all the cases it does not change the status (monogamous or polygamous) of the individual.

Multiple mating and multiple paternity

Nearly half of the females did not produce any eggs (43-50%), while the rest of the females all produced fertilized eggs (Fig. 1). None of the females produced non-hatching eggs only. Multiple matings led to multiple paternity in both experiment 1 and 2. In experiment 1 a high fraction of the mated females (55%) had more than one sire to their offspring. In experiment 2, the offspring were sired by more than one male for a lower fraction (37%) of the mated females. 8-14 % of the males did not sire any offspring (Fig. 2), 14-33% of the males only mated successfully a single time and the majority of males were polygamous (58-73%). In Experiment 1, the most successful male had mated five times, out of 10 possible matings, with an average of 2.3 times per male. In experiment 2, the most successful young and old male had mated five and two times (out of 14 possible) respectively, with an average of 1.2 and 0.6 times per male.

Thus, in both experiments, many females mated successfully with multiple males, and many males mated successfully with multiple females, but mating success was not randomly distributed among individuals ($p = 1.4\ e^{-7}$). Below we explore, for males and females separately, to what extent mating superiority is related to size and age or some other unknown factor.

Male and female reproductive success in relation to body length

Large males were superior to small males in terms of reproductive success, with the larger males (0.8 mm) mating about 3 times as frequent as the smaller males (0.6 mm) (Fig. 3). To investigate the probability that a male of a certain length would mate with a female, we used the GLM model, where we divided the females

in five groups: I) the female with lowest number of matings, II) females with low mating probability (average – 1 SD), III) average, female IV) female with high mating probability (average + 1 SD) and V) the female with most matings. We tested this against male length (Fig. 4). The model shows that male mating success was significantly related to his size ($p < 0.001$), and that there was no further significant difference between males beyond that explained by length ($p=0.9999$). If we compare the five different groups of females, we can see that there is a profound difference in the mating probability between the five groups ($p < 0.001$) (Fig. 4). This difference cannot be explained by female size, as all females in experiment 1 were of the same size. But even in experiment 2, where the females were of different size and with a significant difference in the number of successful matings between females ($p = 0.009$), female mating success was unrelated to her size (Fig. 5). Furthermore, offspring production was unrelated to female size (Fig. 6). There was no significant correlation between body size of females and paired males ($p = <0.001$). Hence there was no evidence for assortative mating for body size in *Temora longicornis.*

Male reproductive success in relation to age

Young males mated more than 3 times a frequent as older males ($p=0.001$, Fig. 7). To test the probability that a male of a certain age (young, old) would mate with a female, we used a model that includes male age and female individual ID as a random effect. The model shows that male age is highly significant ($p=0.001$), with young males having a higher mating probability than old males. Beyond male age, there was no statistically significant mating probability difference between the males ($p>0.99$).

Advantages of multiple matings

Female with multiple sires to their offspring had a higher reproductive output than females that only had one sire to their offspring (Fig. 8), by 59% in experiment 1 and 19% in experiment 2. The effect was statistically significant in experiment 1 and in experiment 1 and 2 combined, but not in experiment 2 alone.

Discussion

The results of this study clearly demonstrated the presence of multiple paternity in *Temora longicornis*. This is the first time in which genetic polyandry has been documented in a pelagic copepod. Until now it has been unclear whether the first attached spermatophore blocked the genital opening of the female, thereby preventing sperm from subsequent attached spermatophores to penetrate and fertilize the eggs (Miller 1988). However, the paternity tests clearly show that the female is capable of using sperm from several males. The existence of simultaneous multiple paternity opens up for post-copulatory sexual selection, such as sperm competition and cryptic female choice (Parker 1970, Olsson et al. 1996, Eberhard 1996, Birkhead 2000, Tregenza & Wedell 2002, Birkhead & Pizzari 2002, Bretman et al. 2004, Simmons 2005). In a range of species, female promiscuity has been demonstrated to be advantageous, by preventing inbreeding (Tregenza & Wedell 2002, Garant et al. 2005), by reducing the risks of genetic incompatibility (Tregenza & Wedell 2002, Dunn et al. 2005, Engqvist 2006), and/or by improving offspring quality through post-copulatory sexual selection. Here we found elevated offspring production in females that mated repeatedly. This is unlikely to be caused by a more plentiful sperm supply because one spermatophore contains sufficient sperm to fertilize all the eggs that a female produces in her life-time (Sichlau & Kiørboe 2011). Our observation rather suggests that post-copulatory sexual selection increases the reproductive output in females with multiple paternities.

In both males and females, mating was non-random. Superior individuals with a higher than average mating success were identified both among females and among males. Some of the variation between individuals could be explained by variation in size and age, but there was also variation that could not be explained by the observed variables. Is this high variation in mating success a laboratory artefact? Only about half of the females in our experiments were fertilized, even though male availability was high and mate encounters not limiting. Observations of the fraction of fertilized female copepods in nature are scarce, however, the few available observations similarly suggest that often only a fraction of the females are fertilized. This holds for the species investigated here (Ceballos et al. 2014), and for the calanoid copepod, *Euchaeta norvegica* (Hopkins 1982, Uye & Sano 1995). In some cases this may be due to a scarcity of males, while in other cases mate encounters are clearly not limiting (Ceballos et al. 2014). A high fraction of unfertilized females is well documented in other invertebrate taxa (Danthanarayana & Gu 1991, Kuussaari et al. 1998, Rhainds et al. 1999, Cueva del Castillo & Nunez-Farfan 2002, Pai et al. 2007). Thus, we conclude that our observations of female mating probability are unlikely to be laboratory artefacts. Knowledge about the fraction of males that actual contributes genes to the future generations in field populations is unknown. We found that a substantial fraction of the males (8-14%) in this study never mated during incubations, despite a plentiful supply of females.

It is optimal for a female to have all her eggs fertilized, so why does such a large fraction of females remain virgins? Similarly, some males are much more successful than others in fertilizing females. We suggest that sexual selection in both sexes through (cryptic) mate choice and/or sperm competition may account for the skewed mating success of both males and females. Three conditions have to be fulfilled to mate choice to be evolutionary successful (Kokko & Monaghan 2001, Shuster 2007). First, mate encounter rate has to be high enough that mates can afford to be choosy. It is known that the mate encounter rate by far exceed the copulatory capacity for the males and the mating needs of the females (Kiørboe 2007), especially since females attract males by producing pheromones (Tsuda & Miller 1998, Strickler 1998, Doall et al. 1998, Bagøien & Kiørboe 2005). Secondly, there must be a significant cost to mating. We know little about the evolutionary trade-offs of mating frequency in copepods. However, multiple mating is known to decrease the longevity of both sexes in other copepod species (Ceballos & Kiørboe 2011). Thirdly, there must be differences within the sexes that can be perceived by the other sex. The results presented here demonstrate that both the male and female can assess different traits (related to quality) of a potential partner.

Mutual mate choice is considered to be rare in nature (Kokko & Johnstone 2002), due to the fact that the conditions that favor one sex to be choosy selects for lack of choosiness in the other sex (Kokko & Johnstone 2002). Mutual mate choice is occurring when the cost of each mating attempt is high for both sexes, there are high encounter rates and both sexes vary in quality (Kokko & Johnstone 2002). Typically, females are expected to be the choosy sex because their reproductive investment is usually larger than males. However, there are indications that the competition for females and the energetic cost of producing spermatophores have larger energetic requirements than commonly believed (Dewsbury 1982). The cost of a spermatophore is much greater than the individual spermatozoa. *Temora longicornis* has a relatively low spermatophore production, < 1.5 spermatophores per day (Ceballos et al. 2014), suggesting that spermatophore production may be expensive, if not in terms of biomass, then maybe in terms of some limiting element. In summary, our results are consistent with mutual mate choice in *Temora longicornis*. Both sexes have a relatively large investment in each mating, we know that the encounter rate by far exceeds the male mating rate and the copulatory needs of the female and that there are quality variation between the individual of both sexes.

The fact that males can mate with more than one female is one of the basics for sexual selection. Thus it may be even more surprising that 14% of the males did not mate successfully, despite a high availability of unfertilized females. This indicates that a fraction of the male population is excluded from the reproductive pool and thus not contributing to the future population. A number of potential factors may explain the unsuccessful males. The lack of mating could be due to failed tracking of the females pheromone trail

(Kiørboe & Bagøien 2005), wrong placement of the spermatophores (Hopkins & Machin 1977, Blades 1977), or that they have mated, but unsuccessfully, due to sperm competition or cryptic female choice occurring in the female's reproductive tract.

Determining the paternity of each offspring allowed us to quantify male reproductive success in relation to two trait; size and age. Our models show that male size and age are important traits for mating, with large and young males having superior in mating success. But the models also showed that female trait have considerable effects on the mating probability. For an average female, the variability in male size implies that mating probabilities range from practically 0 to 0.23 (Fig. 4, line marked III). For comparison, for an average sized male (0.63 mm), variability among females implies that mating probabilities range from practically 0 to 0.91 (Fig., comparing lines I and V for male size 0.63 mm). Similar conclusions can be drawn from the model predicting the probability of mating from male age. The difference in variance in mating probability between young and old males is comparable to the large variance among the female groups. In general, young and large males have greater reproductive success; however the mating probability is controlled by the females rather by the size or age of the male.

Female body size commonly affects the lifetime reproductive success of animals (Clutton-Brock 1988). We did not find a correlation between female size and the number of times she mated. This could be the result of two oppositely directed forces: large females may be the most attractive, but small females may be easier for a male to coerce, this overshadowing any size-dependent variation in attractiveness.

Due to the ecological importance of copepods in aquatic ecosystems, it is critical to understand their population dynamics. Since the future evolution of copepods in a changing environment is determined by differential lifetime fitness of individuals, we need to understand reproductive behaviours in order to predict their genetic diversity and adaptive abilities. For instance, our findings of high variance in reproductive success among individuals indicate that the genetically effective population size (N_e), which is a key parameter in determining levels of genetic diversity as well as response to selection, is much lower than the census size (*see* also Zeller et al. 2008). This study represents a new approach and one of the first steps towards a more comprehensive understanding of mating systems in copepods. The results are promising, however, there is a need to expand this type of analysis to other copepod species and to explore the generality of our results in other mating scenarios.

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Figure legends

Figure 1. Number of sires to the female's offspring. A) Experiment 1, *n*=68, B) Experiment 2, *n* =37.

Figure 2. Number of mating per male. A) Experiment 1, *n* =22, B) Experiment 2, Old males are marked with grey and young males with black, *n* =12.

Figure 3. Number of matings as a function of male prosome length (mm). Experiment 1, *n* = 22.

Figure 4. The probability of male mating, as estimated by the GLM model, as a function of male length for five groups of females. The five groups of females are I) the female with lowest number of matings, II) females with low mating probability (average – 1 SD), III) average, female IV) female with high mating probability (average + 1 SD), and V) the female with most matings.

Figure 5. The percentage of females and the number of sires to offspring in each size group. Small females 0.74-0.80 mm, medium females 0.80-0.87 mm, and large females 0.87-0.94 mm. Experiment 2, *n* = 37. The differences between groups are not statistically significant (Fisher´s exact test for count data, p = 0.95).

Figure 6. Number of nauplii produced in relation to female prosome length (mm). Experiment 2, *n* = 21.

Figure 7. The number of successful mating in the two age groups. Young males are matured within the last 24 hours and old males are >5 days. Experiment 2, *n* = 12. The differences between groups are statistically significant (t-test, $p < 0.001$).

Figure 8. Nauplii per female during the first five reproductive days. A) Experiment 1, *n* =29 (13 single paternity, 16 multiple paternity), B) Experiment 2, *n* =16 (8 single paternity, 10 multiple paternity). In experiment 1 the difference between the reproductive output from females that have mated single and multiple times is statistically significant ($p > 0.001$), while that difference is not significant in experiment 2 ($p = 0.115$). When combining the two dataset, there is a significant difference between the two groups ($p > 0.001$).

Table 1

Polymorphism of the five microsatellite loci over 45 adults (24 females and 21 males) used for the paternity analysis. Locus name, number of observed alleles (N_a), expected heterozygosity (H_e), observed heterozygosity (H_o), polymorphism information contents (PIC) and the probability value of an exact test for the null hypothesis of Hardy-Weinberg equilibrium (P_{HW}) are indicated for each locus and over all 5 loci used.

Locus	**N_a**	**H_E**	**H_o**	**PIC**	**P_{HW}**
Tl01	7	0.722	0.658	0.680	0.036
Tl06	14	0.797	0.825	0.776	0.336
Tl07	2	0.219	0.205	0.195	0.516
Tl08	5	0.448	0.415	0.413	0.497
Tl11	4	0.696	0.779	0.638	0.691
Over all loci	6.4	0.576	0.576	0.540	0.415
Standard deviation	4.6	0.239	0.261	0.210	

FIGURE 1

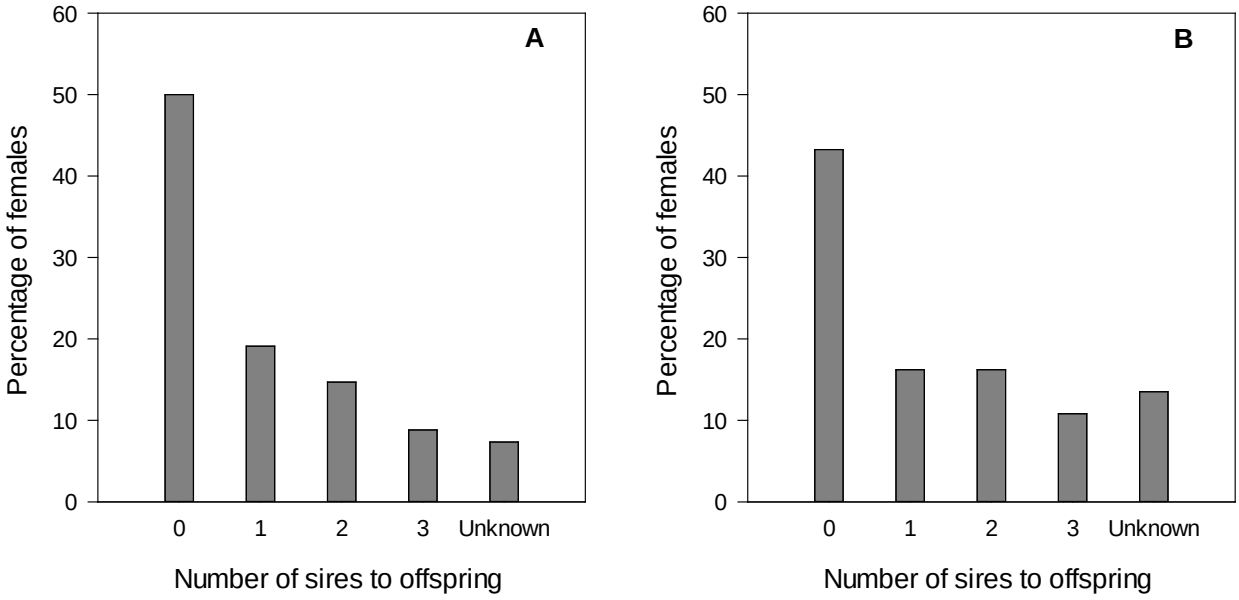

A
B
Percentage of females
0
10
20
30
40
50
60
0
1
2
3
Unknown
Number of sires to offspring

FIGURE 2

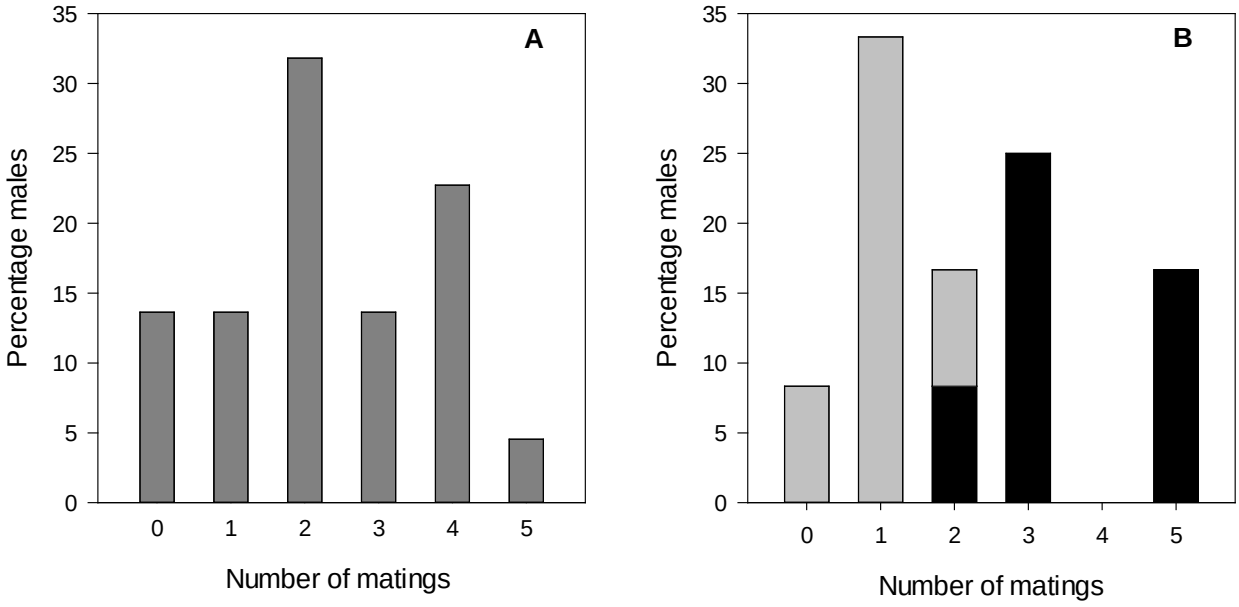

A
B
Percentage males
Number of matings
0
5
10
15
20
25
30
35
0
1
2
3
4
5

FIGURE 3

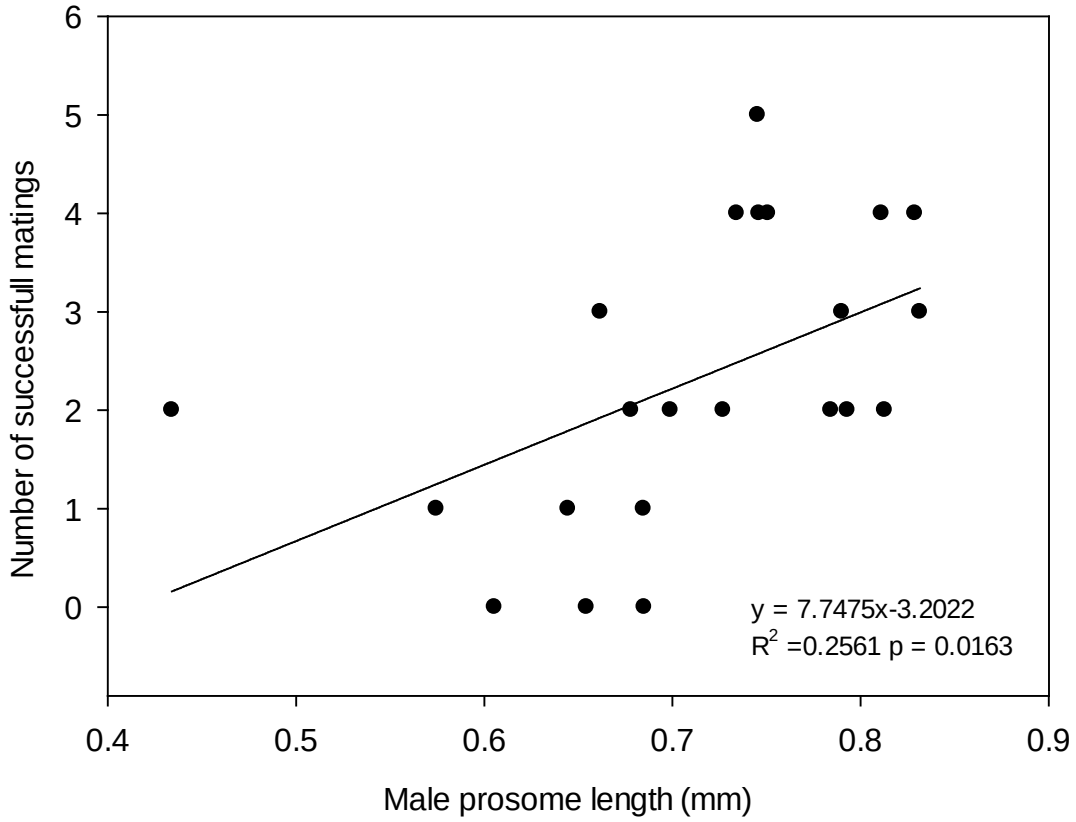

Number of successfull matings
Male prosome length (mm)
y = 7.7475x-3.2022
R2 =0.2561 p = 0.0163
0
1
2
3
4
5
6
0.4
0.5
0.6
0.7
0.8
0.9

FIGURE 4

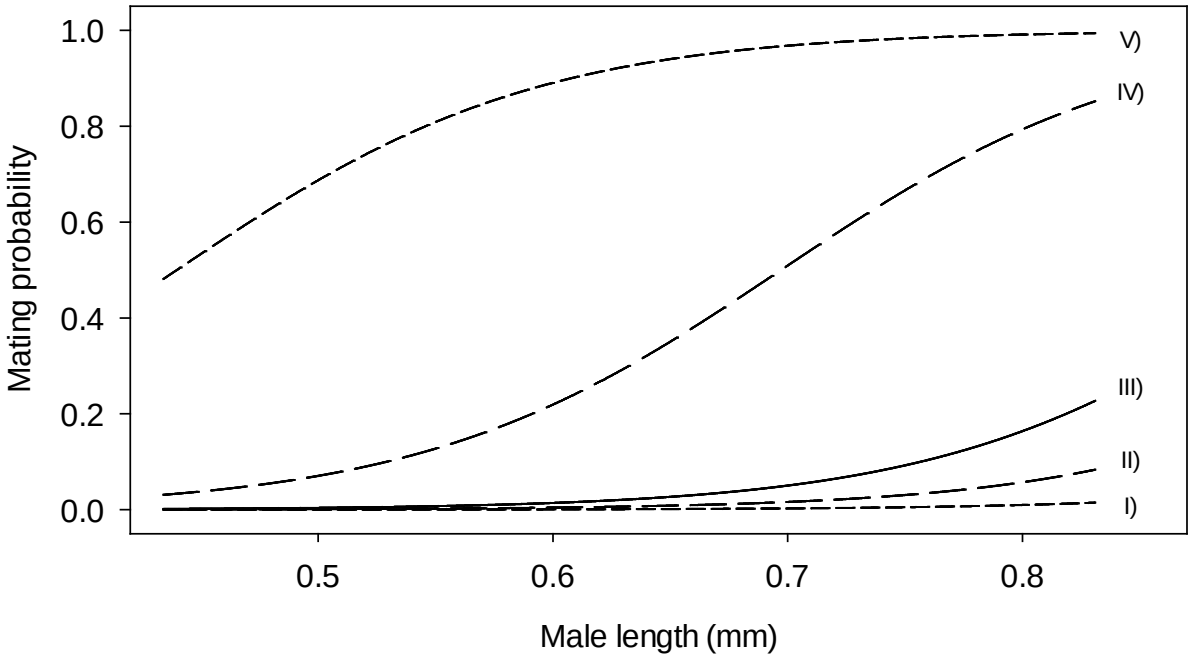

1.0
0.8
0.6
0.4
0.2
0.0
Mating probability
0.5
0.6
0.7
0.8
Male length (mm)
V)
IV)
III)
II)
I)

FIGURE 5

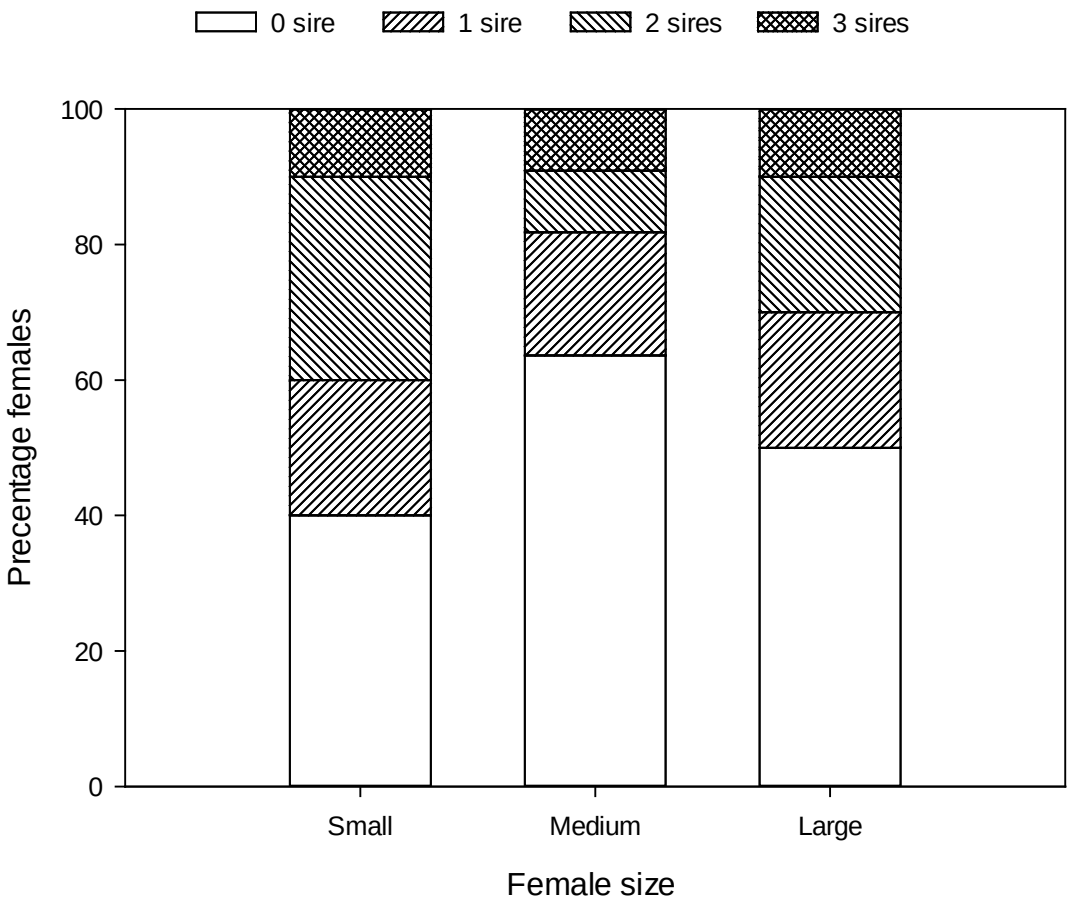

0 sire
1 sire
2 sires
3 sires
100
80
60
40
20
0
Precentage females
Small
Medium
Large
Female size

FIGURE 6

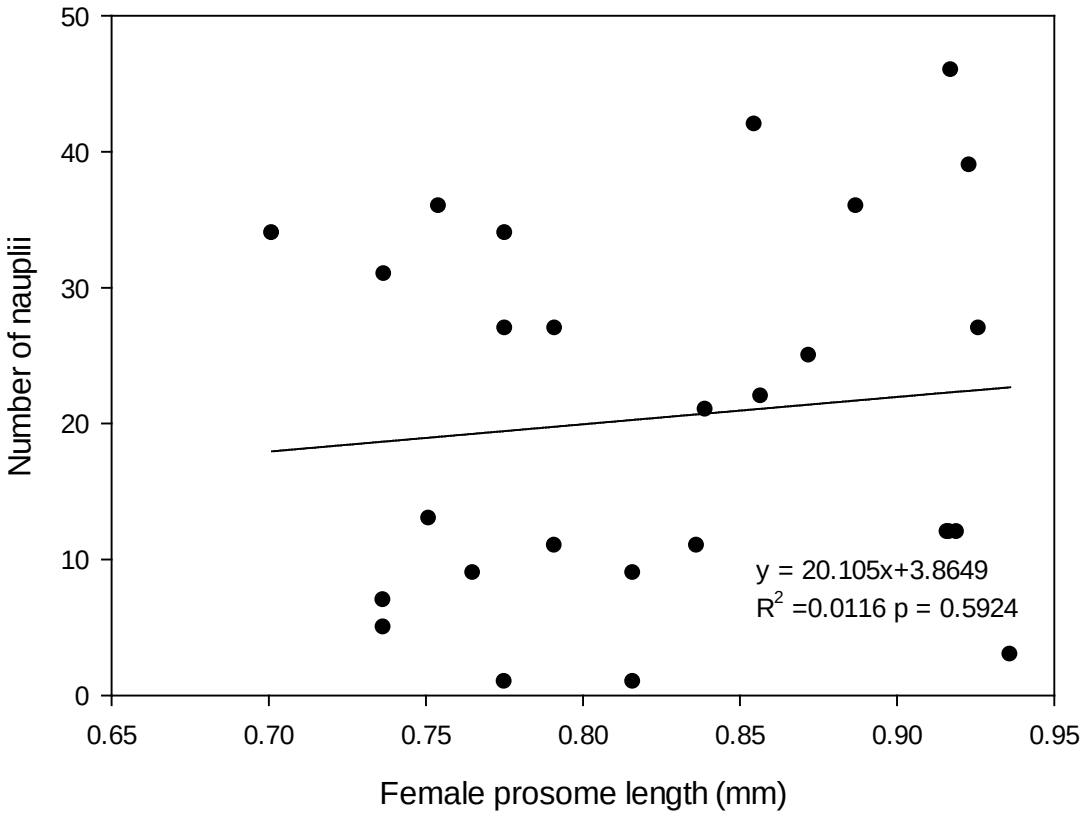

Number of nauplii
Female prosome length (mm)
y = 20.105x+3.8649
R2 =0.0116 p = 0.5924
0
10
20
30
40
50
0.65
0.70
0.75
0.80
0.85
0.90
0.95

FIGURE 7

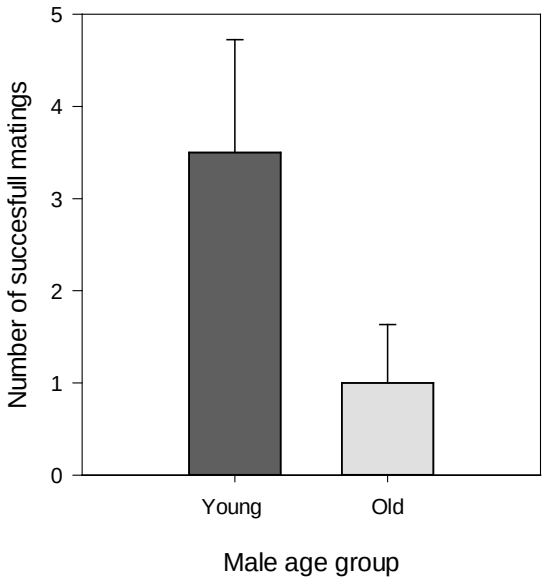
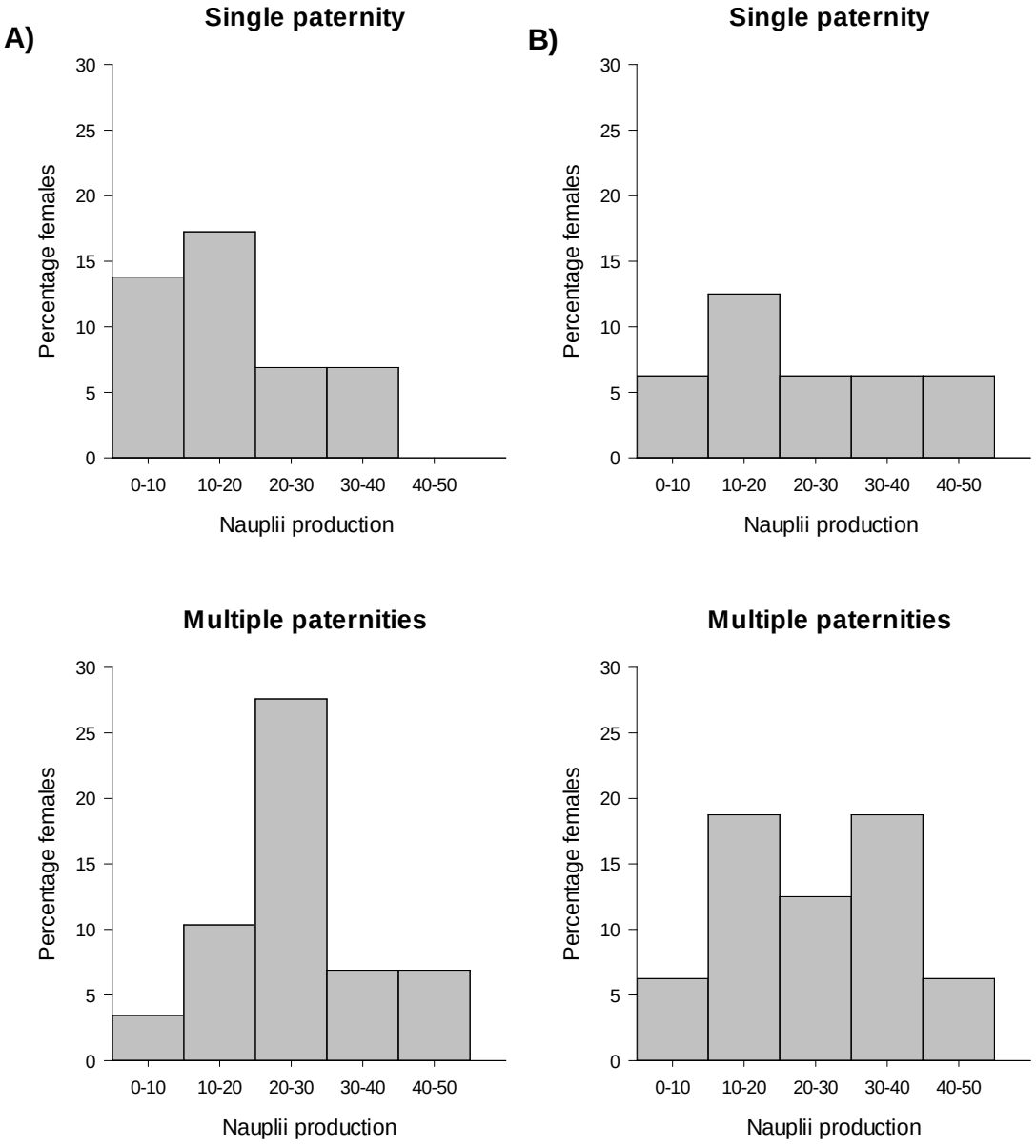

Number of succesfull matings
5
4
3
2
1
0
Young
Old
Male age group

FIGURE 8


A)
Single paternity
Percentage females
0
5
10
15
20
25
30
0-10
10-20
20-30
30-40
40-50
Nauplii production
B)
Single paternity
Percentage females
0
5
10
15
20
25
30
0-10
10-20
20-30
30-40
40-50
Nauplii production
Multiple paternities
Percentage females
0
5
10
15
20
25
30
0-10
10-20
20-30
30-40
40-50
Nauplii production
Multiple paternities
Percentage females
0
5
10
15
20
25
30
0-10
10-20
20-30
30-40
40-50
Nauplii production

DTU Aqua – National Institute of Aquatic Resources – is an institute at the Technical University of Denmark. DTU Aqua's mission is to conduct research, provide advice, educate at university level and contribute to innovation in sustainable exploitation and management of aquatic resources. We investigate the biology and population ecology of aquatic organism, aquatic physics and chemical pro-cesses, ecosystem structure and dynamics, taking account of all relevant natural and anthropo-genic drivers.

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